Universidade de Aveiro Departamento de Biologia
Ano 2013

Ana Rita Marques Almeida

Efeito combinado da radiação UV e xenobióticos no peixe zebra

Combined effects of Ultraviolet radiation and xenobiotics on zebrafish

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada no ramo Microbiologia Clínica e Ambiental, realizada sob a orientação científica da Doutora Paula Inês Borralho Domingues, Bolseira de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro, co-orientação do Doutor Newton Carlos Marcial Gomes, Investigador auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

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Agradecimentos

Agradeço:

À minha orientadora Dr.ª Inês Domingues por todo apoio, motivação, paciência e ajuda dada ao longo de todo o trabalho. Foram muito importantes.

Ao meu co-orientador Newton Gomes por toda a disponibilidade e sugestões dadas.

À minha família, pai e mãe, por todo o esforço, amor e dedicação para que conseguisse terminar esta etapa.

Ao João pela companhia, amor e incentivo dado ao longo de todos estes anos.

À Dr.ª Susana Loureiro por toda a ajuda e sugestões dadas.

À Tânia pela paciência, ajuda e todas as horas "perdidas" a tentar ensinar-me. Agradeço-lhe muito por isso.

Ao pessoal dos "peixes", Thayres, Jorge e Violeta por toda a ajuda, companhia, boa disposição e trocas de conhecimentos.

A todo o pessoal do LEMAN, Ana, Patrícia, Rossana, Rita, Francisco e Fernando por toda a ajuda e incentivo.

E finalmente, agradeço aos meus amigos e a todas as pessoas que ao longo destes anos passaram pela minha e deixaram a sua marca.

Palavras-chave

Peixe-Zebra, Xenobióticos, comunidades bacterianas naturais, efeitos combinados.

Resumo

Hoje em dia, as alterações climáticas são um problema imperativo e múltiplas medições feitas nos últimos anos mostram um aumento de toda a radiação solar, especialmente a radiação Ultravioleta que chega á superfície da terra afetando todos os organismos expostos. No seu ambiente natural, os organismos não estão apenas sujeitos a fatores bióticos, mas também a fatores ambientais e abióticos como por exemplo os xenobióticos. Além disso, ambos os stressores podem interagir uns com os outros produzindo efeitos imprevisíveis nos organismos (efeitos sinergísticos ou antagonísticos). O presente trabalho tem como objetivo a avaliação dos efeitos combinados da radiação UV e três xenobioticos (triclosan, dicromato de potássio e procloraz) em embriões de peixe zebra (*Danio rerio*). A avaliação foi feita a dois níveis: i) efeitos na mortalidade de embriões e ii) efeitos a nível das comunidades bacterianas naturais dos embriões. Os organismos foram expostos a várias concentrações de cada químico, combinadas com várias doses de UV. A mortalidade foi registada diariamente durante 96 horas e as comunidades bacterianas naturais foram avaliadas às 48 horas pós fertilização (hpf). Os resultados mostram que diferentes efeitos combinados foram observados, alterando a ecotoxicidade esperada. A exposição combinada da radiação UV com o TCS revelou um patrão sinergístico quando a radiação UV é o stressor dominante, enquanto que, na combinação UV com PD e PCZ observou-se antagonismo a doses baixas ou quando a radiação UV era dominante na mistura. As comunidades bacterianas naturais do peixe zebra também foram afetadas pela radiação UV e químicos, com alterações na sua estrutura. No entanto, foi difícil tirar conclusões relativamente a possíveis interações entre stressors visto que os efeitos observados nem sempre se traduziam em variações no índice de diversidade.

Key Words

Zebrafish, Xenobiotics, Natural bacterial communities, combined effects.

Abstract

Nowadays, climate changes are an imperative problem and multiple measurements made in the last years showed an increase of all wavelengths of solar radiance, specially the Ultraviolet radiation. In their natural environment organisms are not only affected by biotic and environmental factors, but also by abiotic factors such as xenobiotics. Besides, these both stressors can interact with each other being their combined effect unpredictable (producing additive, synergistic or antagonistic effect). This work aims to studying the combined effect of UV radiation and three xenobiotics: triclosan, potassium dichromate and prochloraz on zebrafish embryos (*Danio rerio*). Effects were assessed at two levels: i) effects on embryos mortality and ii) effects in the natural bacterial communities of zebrafish embryos. The organisms were exposed to concentrations of each chemical combined with several UV doses. Embryo' mortality, were observed daily for 96 hours post fertilization (hpf) and natural bacterial communities' evaluation was performed at 48 hpf. Results showed that different combined effect may occur compromising organism's survival. Combined exposure of UV radiation with TCS revealed a synergism pattern when the UV radiation is the dominant stressor while PD and PCZ revealed antagonism at low dose levels or when the UV radiation is dominant in the mixture. Zebrafish natural bacterial communities were also affected by UV radiation and chemicals with the change of their structure; however, conclusions about interactive effects were difficult to be drawn because effects were not always translated into changes in the diversity indexes.

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Chapter 1

1. INTRODUCTION

1.1. Climate changes

Climate can be expressed by the variation of temperature, precipitation and other physical properties, or in other words, by the typical meteorological conditions of a particular local for a certain period of time (Posas, 2011; Wuebbles, Jain, Edmonds, Harvey, & Hayhoe, 1999).

In the last two decades of the 20th century, an overall warming was becoming noticed with surface temperatures increasing 0,16 % per decade (Lean, 2010). This effect can be in part explained by anthropogenic influences, such as the emission of greenhouse gases, chlorofluorocarbons (CFCs) and other chlorinated and brominated halocarbons substances. While greenhouse gases absorb infrared radiation, turning the earth surface warmer, CFCs lead to ozone depletion. Given that ozone has an important role in filtering the ultraviolet (UV) radiation (Biales, Bencic, Villeneuve, Ankley, & Lattier) its depletion leads to increasing radiation reaching the earth' surface (Lean, 2010; McKenzie et al., 2011; Wuebbles et al., 1999). Consequently, both gases contribute for changing the radiative balance of the atmosphere and modify the rate of warming and cooling of the earth. In fact, climate changes and ozone depletion are interconnected, variations in ozone layer can produce changes in climate and reciprocally. Nevertheless, UV radiation can be changed by climate changes without affecting ozone layer (McKenzie et al., 2011; Wuebbles et al., 1999).

Decreasing the greenhouse gases and ozone depleting substances (ODSs) emissions became essential to face the rise of temperature and UV radiation on earths' surface. Therefore two international agreements were created to ban the use of CFCs (Montreal Protocol) and limit the emission of greenhouse gases (Kyoto protocol) (Holtsmark & Mæstad, 2002; Partridge, 1998).

Evidences show that Montreal Protocol was successful and the ozone layer is recovering. However, UV radiation reaching the surface of the earth is not decreasing (McKenzie et al., 2011; Woodcock, 2009). Indeed, solar activity tends to increase in the recent centuries and multiple measurements that have been made in the last years show an increase of all wavelengths of solar radiance, especially middle UV radiation (Lean, 2010; Lean & DeLand, 2012).

1.1.1. Ultraviolet radiation

Ultraviolet radiation is the radiation comprised between 100 and 400 nm being an important environmental stressor for both aquatic and terrestrial organisms (Nazari et al., 2010; Tartarotti & Torres, 2009). It can be divided in three categories: UV-A (400-315nm), UV-B (315-280 nm) and UV-C (280-100 nm) (Dong, Svoboda, Tiersch, & Monroe, 2007). The UV-C radiation is mostly absorbed by atmospheric ozone, and has not a significant environmental effect. On the other hand, UV-A and UV-B radiation reach the surface of the earth, having a higher impact in the environment (Dahms, Dobretsov, & Lee, 2011; Dahms & Lee, 2010).

Over the last years, many studies have revealed the harmful effects of UV-A and UV-B radiation. The main consequence of UV radiation is the generation of reactive oxygen species (ROS) that will damage cellular macromolecules (Behrendt, Jonsson, Goldstone, & Stegeman, 2010). Furthermore, proteins, lipids and nucleic acids are the first target of UV radiation, causing damages that can compromise common cellular process (Dahms et al., 2011).

In the aquatic ecosystems, the penetration of UV radiation depends on the dissolved organic carbon and colored non-living organic matter, being higher in cleaner water (Behrendt et al., 2010; Häkkinen et al., 2002; Sayed Ael, Ibrahim, Mekkawy, & Mahmoud, 2007). Decreases in productivity of phytoplankton and zooplankton by UV influence have been reported in literature. This variation can affect community composition by altering their diversity and productivity (Dahms et al., 2011).

In fishes, studies have shown a decrease of both growth and metabolic rate and even an effect on swimming performance (Sayed Ael et al., 2007). In several species egg mortality increases when exposed to UV radiation. In the adults, UV radiation acts like an immunosuppressive agent, destroying the immune systems including blood cells (Häkkinen et al., 2002; Sayed Ael et al., 2007).

On the other hand, some organisms that are subjected to high intensities of UV radiation have developed protection mechanisms. Sommaruga (2001) showed some strategies of alpine organisms, such as the melanization of dorsal portion in *Daphnia spp*., the accumulation of carotenoids in some copepods, which can minimize ROS production, the production of scytonemin pigment, which is present only in cyanobacteria, in particular those that live under intense solar radiation condition, and mycosporine-like amino acids. However, these strategies are not present in all organisms, being aquatic organisms from environments at lower altitudes more sensitivity to UV radiation (Sommaruga, 2001).

1.2. Xenobiotic compounds

Xenobiotics are compounds prevenient from diverse human activities that reach the environment posing serious risks to organisms and ecosystems. These compounds, such as pesticides, pharmaceuticals and nanoparticles do not occur in the environment naturally. Natural elements, such as compounds released from a forest fire or mining activities, which would usually be present in the environment only in vestigial concentrations, are also considered xenobiotics (Chong & Huang, 2012; Scott & Sloman, 2004). Xenobiotics have a wide range of physico-chemical properties and derive from numerous applications. Actually, much time and money is spent in developing new substances with the appropriate feature for a particular use, mostly because society is highly dependent on supplies and activities that involve the use of artificial synthetized compounds or naturals elements that are not so abundant on the surface of the earth (Donner et al., 2010).

In general, organisms can degrade xenobiotics after they enter in the body (biotransformation) converting them to less toxic and more easily to excrete products. However, in some cases this process can produce metabolites with higher toxicity than the original compound (Rivière, Bach, & Grolleau, 1985; Scott & Sloman, 2004; Sturm, Cravedi, Perdu, Baradat, & Segner, 2001). Further, the increase of reactive oxygen species levels is a common cause of xenobiotic exposure, leading to an oxidative stress, cellular damage and tissue injury. Furthermore, behavioral disruption (i.g. reproducibility and predator avoidance) and physiological response (i.g. neurobiological, endocrine and hormonal dysfunction) are some of the effects observed in fish after chemical exposure (Figure 1) (V. I. Lushchak, 2011; Scott & Sloman, 2004).

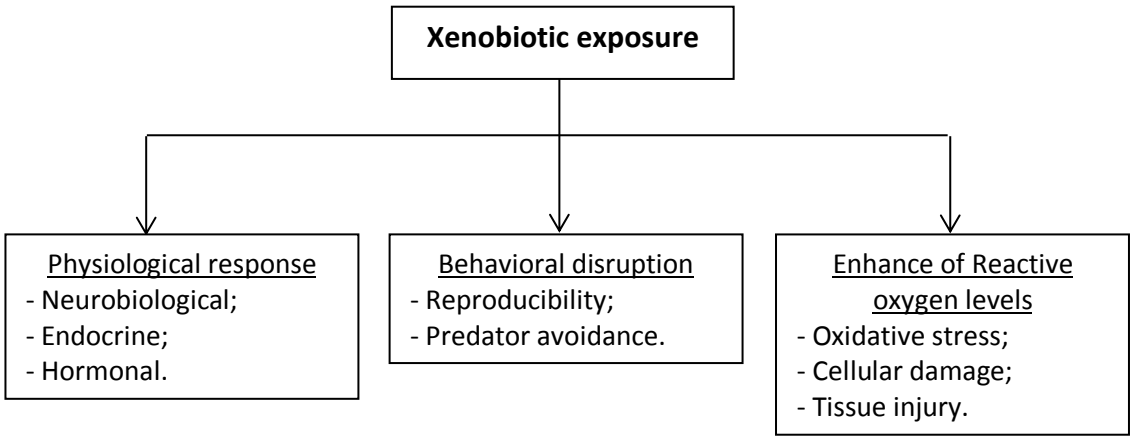

Figure 1- Xenobiotic exposure effects adapted fromScott and Sloman (2004)

Triclosan [5-chloro-2-[2,4-dichloro-phenoxy]-phenol; TCS] is a broad spectrum antimicrobial compound created 40 years ago (Fig. 2) (Dann & Hontela, 2011; Orvos et al., 2002). In the last 25 years, their use has been increasing once it is becoming part of many contemporary products such as: hand soaps, shower gels, deodorant soaps, screen cream, toothpaste, plastics and some textiles (Dann & Hontela, 2011; Liu, Ying, Yang, & Zhou, 2009; Lubarsky et al., 2012).

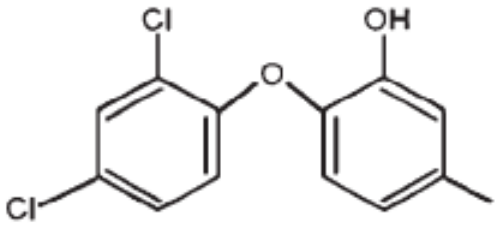

Figure 2 - Chemical structure of Triclosan (Dann & Hontela, 2011)

TCS is deposed with domestic wastewater, arriving later to treatment station, however, studies show that wastewater treatment plant are not able to remove completely this compound. Thus, TCS has already been detected in surface water, sediments, soils and in aquatic species (Dann & Hontela, 2011; Orvos et al., 2002; Sankoda et al., 2011). In fact, this may be the primary route for TCS enter in environment. Some predictions indicate, an increasing detection of the TCS in waterways once antimicrobial products are largely used (Dann & Hontela, 2011; Liu et al., 2009).

Some studies have shown that TCS affects negatively a large variety of aquatic organisms. Algae are an important pathway of accumulation of this compound once they are the primary food source for many species and can affect the whole aquatic ecosystem. Moreover, literature revealed bioaccumulation of TCS on fish (Dann & Hontela, 2011; R. Oliveira, Domingues, Grisolia, & Soares, 2009). Effects induced by TCS on adult fishes are loss of equilibrium, blockage of the mouth, quietness and irregular swimming movements (Orvos et al., 2002). Acute toxicity was also showed by Oliveira et al (2009) on zebrafish early-life stages as spinal malformations, hatching delayed, abnormal pigmentation of the eye and body and undersized larvae.

1.2.1. Potassium Dichromate

Chromium is abundant heavy metal widely distributed in terrestrial and aquatic environments (Farag et al., 2006; O. V. Lushchak, Kubrak, Nykorak, Storey, & Lushchak, 2008; Mohan & Pittman Jr, 2006). Similarly to the others metals, this compound is not

biodegradable and represents an important environmental threat once it remains in ecosystems and can be accumulated through the food chain (El Nemr, El-Sikaily, Khaled, & Abdelwahab; O. V. Lushchak et al., 2008).

This compound occurs in 2+ [bivalent form, Cr(II)], 3+ [trivalent form, Cr (Erickson Iii, Zepp, & Atlas)] and 6+ [hexavalent form, Cr(VI)] oxidative forms. The 2+ form is not abundant in nature, is unstable and little is known about its hydrolysis. Cr 3+(Erickson Iii et al.) is a hard acid, insoluble, immobile in ambient circumstances and can form strong complexes with oxygen and other donor ligands (Barnhart, 1997; Mohan & Pittman Jr, 2006). The hexavalent form has higher water solubility and mobility what makes it more toxic than the others oxidative forms of Cr (Erickson Iii et al.). Indeed, due to their properties, Cr(VI) crosses readily the cell membrane and, once inside of the cell, it is reduced to Cr (Erickson Iii et al.) which form complexes with intracellular macromolecules as well as with genetic material. Chromate and dichromate are the most toxic forms of hexavalent chromium (Farag et al., 2006; Mishra & Mohanty, 2009; Mohan & Pittman Jr, 2006).

Chromium compounds are used by several industries such as textile, wood preservations, corrosion control, electroplating, metal finishing industries, stainless steel productions, etc. Frequently, hexavalent chromium is discarded with other untreated industrial effluents reaching the water surface. Actually, global discharge in water of chromium is 142000 metric tonnes by year (Mishra & Mohanty, 2009; Mohan & Pittman Jr, 2006).

In the last decades, several studies have been showing the toxicity of chromium, to aquatic organisms, especially fishes (Mishra & Mohanty, 2009). In the mudskipper *Perophthalmus dipes*, some enzymes of gills, kidney and intestine were inhibited by chromium. (O. V. Lushchak et al., 2008) Moreover, metabolic changes in the Indian major carp *Labeo rohita*, and alterations of growth and health parameters in Chinook salmon, *Oncorhynchys tshawytscha*, and mummichog, *Fundulus heteroclitus* were also described as effects of chromium (Farag et al., 2006; Roling, Bain, Gardea-Torresdey, Bader, & Baldwin, 2006; Vutukuru, 2003). Moreover, immune response and resistance to disease of tilapia, *Oreochromis mossambicus*, was suppressed by chromium exposure (Prabakaran, Binuramesh, Steinhagen, & Michael, 2006).

1.2.2. Prochloraz

Procloraz (N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]-1H-imidazole-1-carboxamide) (Fig. 3) is a broad spectrum fungicide that pertains at imidazole family. The base of its action is the inhibition of cytochrome P450, essential in the biosynthesis of ergosterol, a fundamental component in the fungal membrane. (Biales et al., 2011; Kinnberg, Holbech, Petersen, & Bjerregaard, 2007)

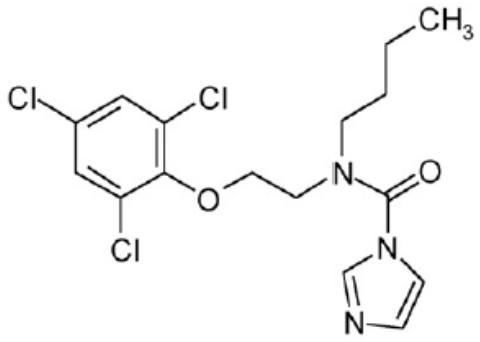

Figure 3 – Chemical structure of Prochloraz (Ohlsson, Ullerås, & Oskarsson, 2009).

Since the early 1970s, prochloraz has been widely used in horticulture and agriculture. Its main application is to reduce plant disease, inhibiting fungi metabolism. Nevertheless, due to its broad spectrum properties, it affects also non target organisms, and may decrease microbial diversity and soil fertility (Ohlsson et al., 2009; Tejada, Gómez, García-Martínez, Osta, & Parrado, 2011)

Effects of this compound have been documented by a number of researches. Indeed, prochloraz affects steroid hormone levels in fetuses and/or adults of Wister dams, interfere with the receptors of androgen (Ohlsson et al., 2009; Vinggaard et al., 2006) and also alter hepatic and microsomal monooxygenases activity of Japanese quail (Rivière et al., 1985). In fishes, it interferes with metabolic pathway (e.g. biotransformation process), and produces behavioral disorders such as changes on swimming patterns, loss of equilibrium and reduced activity (Domingues et al., 2011; Saglio, Bretaud, Rivot, & Olsén, 2003).

1.3. Stressors interactions in natural ecosystems

In their natural environment, organisms are subjected to a multiplicity of stressors, which can interact with each other (Beketov, Speranza, & Liess, 2011; Tu et al., 2012). Moreover, these interactions are unpredictable, and can produce synergistic effects (when the interaction produces higher effect than the sum of the individual), or an antagonistic effect (when the interaction produces smaller effect than the sum of the individual) (Szöcs, Kefford, & Schäfer, 2012).

Several studies have been made in order to understand the effects of singular chemicals in aquatic environments, however, few is known about possible interactions with environmental parameters. Climate changes, and specially, the increase of UVR, has highlighted the importance of including environmental factors as additional stressors in ecotoxicological tests (Beketov et al., 2011).

In the last two decades, some studies showed significant interactions of temperature, salinity and toxicants in grass shrimp *(Palaemonetes pugio)*, in zooplankton, in rotifer *(Brachionus rotundiformis)* and in black tiger shrimp *(Penaeus monodon)* and worms (*Enchytraeus albidus*) (Gama-Flores, Sarma, & Nandini, 2005; Howard & Hacker, 1990; Silva, Holmstrup, & Amorim, 2013; Tu et al., 2012). The toxicity of metals and pesticides with the increasing of temperature was also showed by several studies (Table 1 and 2).

Moreover, the combined toxicity of metals and dissolved oxygen was showed by Ferreira et al. (2008) and Ferreira et al. (2010) revealing the enhancing of cadmium and nickel toxicity respectively at low dissolved oxygen levels in *Daphnia magna*.

Table 1- Interaction studies between temperature increasing and metals (adapted from Holmstrup et al. (2010))

Metal	Test Organism	Species	Interaction	Reference
Cadmium	Mussel	*Grassostrea virginica*	Synergistic	Lannig, Cherkasov, and Sokolova (2006)
	Woodlouse	*Porcellioscaber*	Antagonistic	Abdel-Lateif, Donker, and Van Straalen (1998)
	Fish	*Danio rerio*	Synergistic	Hallare, Schirling, Luckenbach, Köhler, and Triebskorn (2005)
Nickel	Water flea	*Daphnia magna*	Synergistic	Ferreira et al. (2010)
	Fish	*Danio rerio*	Synergistic	Scheil and Köhler (2009)
Mercury	Springtail	*Folsomia candida*	Synergistic	Slotsbo et al. (2009)
Copper	Water flea	*Daphnia pulex*	Synergistic	Boeckman and Bidwell (2006)
	Fish	*Ictalurus punctatus*	Antagonistic	Perschbacher (2005)
Zinc	Earthworm	*Lumbricus terrestris*	Synergistic	Khan et al. (2007)
Lead	Crayfish	*Orconectes immunis*	Synergistic	Khan et al. (2006)

Table 2- Interaction studies between temperature increasing and pesticides (adapted from Holmstrup et al. (2010)).

Pesticides	Test Organism	Species	Interaction	Reference
Fenarimol	Amphipod	*Monoporeia affinis*	Synergistic	Jacobson, Prevodnik, and Sundelin (2008)
Diazinon	Fish	*Danio rerio*	Synergistic	Osterauer and Köhler (2008)
Chlorpyrifos	Insect	*Chironomus tentans*	Synergistic	Lydy, Belden, and Ternes (1999)
Imidachloprid	Fish	*Danio rerio*	None	Scheil and Köhler (2009)
Terbufos	Fish	*Cyprinodon variegatus*	Synergistic	Brecken-Folse, Mayer, Pedigo, and Marking (1994)
Trichlorfon	Shrimp	*Palemonetes sp.*	Synergistic	Brecken-Folse et al. (1994)Brecken-Folse, Mayer, Pedigo, and Marking (1994)
M-parathion	Insect	*Chironomus tentans*	Synergistic	Lydy et al. (1999)
Pentachlorobenzene	Insect	*Chironomus tentans*	Synergistic	Lydy et al. (1999)
Profenofos	Fish	*Pimephales promelas*	Synergistic	Baer, Olivier, and Pope (2002)
Pyrethrine	Lizard	*Anolis carolensis*	Antagonistic	Talent (2005)

1.3.1. Ultraviolet radiation and toxicants interaction

It would be expected that interactions between UVR and toxicants led to an additive effect or synergistic effect, once UVR can produce chemical changes by photoactivation, resulting in an increase of toxicity (Beketov et al., 2011).

Indeed, this enhanced toxicity was demonstrated by Huovien et al. (2001) with polycyclic aromatic hydrocarbons and by Jung et al. (2008) with sulfonamide antibiotics in *Daphnia magna*. Moreover, synergism was also showed by several studies on these organisms. Ribeiro et al (2011) observed synergism patterns on exposure at UV radiation and carbendazim, when the toxicity of combination was produced mainly by UVR. Kim et al. (2009) showed an increase of oxidative stress when *D.magna* were exposure to a different UV-B levels and sulfathiazole and, Nikillä et al. (1999) observed also an enhanced toxicity of pyrene under UV-B radiation.

1.4. Natural Microflora

Microorganisms are the most abundant form of life on the planet playing a critical rule, providing essential services that make our planet habitable (Table 3) (Li, Yu, Feng, Yan, & Gong, 2012; Prakash, Shouche, Jangid, & Kostka, 2013; Whitman, Coleman, & Wiebe, 1998).

Table 3 – Perspective of prokaryotes abundance in the world (Whitman et al., 1998)

Environment	**No. of prokaryotes cells (x10^{28})**
Aquatic habitats	12
Ocean surface	355
Soil	26
Terrestrial surface	25-250

Over their life, animals have living in a microbial rich environment where they constitute intimal associations with microbial communities. (Yan, van der Gast, & Yu, 2012) Every single organism have its own microflora, in other words, is colonized by several bacteria types that live in a symbiotic association with the host. This bacterial composition may consist on aerobic, facultative anaerobic and obligate anaerobic microorganisms. (Fraune & Bosch, 2010; Ringo, Olsen, Mayhew, & Myklebust, 2003)

The majority of these microorganisms is situated in the digestive tract and plays an important rule on hosts' life (Kanther & Rawls, 2010; Yan et al., 2012). Natural microflora contributes to the host immune system, being the "first line defense" against pathogens, further, they furnish some nutrients that host could not access alone, such as vitamins, minerals and enzymes. Cell renewal in the intestine epithelium is also stimulated by these microrganisms (G. H. Hansen & Olafsen, 1999; Ozaktas, Taskin, & Gozen, 2012; Yan et al., 2012). Some authors have already shown the benefits of microflora, such as the preventing of infection by pathogenic fungi in crustacean eggs by bacteria producing an antifungal compound (Gil-Turnes & Fenical, 1992), the weight regulation by gut microbiota in mice and humans (Fraune & Bosch, 2010; Serino, Luche, Chabo, Amar, & Burcelin, 2009) and behavioral patterns monitoring in mammals (e.g. individuals, group members and parents recognition by odor and mate choice by producing secondary sexual traits) (Archie & Theis, 2011).

The natural microflora is specific of each organism, being different between species and inside of the same species. In fishes, microbiota is constituted mainly by four phyla: *Proteobacteria*, *Fusobacterium*, *Actinobacteria* and *Cyanobacteria* (Li et al., 2012; Roeselers et al., 2011a). Several studies have shown the differences between fish species microflora (Table 4).

Table 4 – Fish natural microflora

Organism	Specie	Microflora	Reference
Coho salmon	*Oncorhynchus kisutch*	Pseudomonas sp.; Aeromonas sp.; Vibrio sp.	Romero and Navarrete (2006)
Salmon	*Salmo salar L.*	Pseudomonas fluorescences; Acinetobacter sp.	Navarrete, Espejo, and Romero (2009)
Rainbow trout	*Oncorhynchus mykiss*	Proteobacteria	Kim, Brunt, and Austin (2007)
Halibut	*Hippoglossus hippoglossus L.*	Pseudoalteromonas sp. Marinomonas sp. Vibrio sp.	Bjornsdottir et al. (2010)

Natural microflora can change with age, nutritional status and environment conditions (Ringo et al., 2003). Some environmental stressors such as temperature, oxygen levels, pH or pollutants can have a negative influence weakening these "first line defense" of the organism. Moreover, fitness of organisms may also be compromised which can also can change bacteria virulence (G. H. Hansen & Olafsen, 1999; Li et al., 2012).

1.4.1. Early life stages microflora

Vertebrates are born free of microrganisms, being colonized just in few hours (Yan et al., 2012). Indeed, the surface of eggs and larvae skin are good substrate for bacteria adhesion (Geir Høvik Hansen & Olafsen, 1989). This initial colonization is highly influenced by the surrounding environment and it is made by non-opportunistic bacteria that constitute the established microflora and will protect the host from pathogenic bacteria (Huys et al., 2001).

At egg stage of development, the eggshell is constituted by zona radiata and chorion (Fig. 4). This structure is mainly constituted by glycoproteins which make this place suitable for bacterial adhesion. Further, the embryo is able to secret some organic and

inorganic compound, producing a gradient which will act as a chemo-attractant for specific bacteria (G. H. Hansen & Olafsen, 1999). Some receptors present in the egg surface also act as bacteria selective (Olafsen, 2001).

Figure 4- Zebrafish egg

The egg adherent epiflora is highly diverse and act mostly has a barrier against pathogens present in the surrounding environment (G. H. Hansen & Olafsen, 1999). Some studies have shown the importance of natural microflora where these symbiotic interactions contribute to the survival of larvae (Brunvold et al., 2007). Moreover, various fish eggs are colonized by pigmented bacteria that constitute a protection against UV-B radiation (e.g. cod and halibut) (G. H. Hansen & Olafsen, 1999).

After hatching, fish larvae acquire its own/indigenous microflora when they start drinking and consume the suspended bacteria in the water. This happens before the yolk sac is consumed and establishes the primary intestinal microbiota (G. H. Hansen & Olafsen, 1999; Jensen, Øvreås, Bergh, & Torsvik, 2004; Olafsen, 2001). However, the main diversity and quantity of bacteria is uptake when the larvae start feeding (Fjellheim, Playfoot, Skjermo, & Vadstein, 2012).

1.5. Zebrafish

The zebrafish, *Danio rerio*, was firstly described by Hamilton-Buchanan in 1822 and belongs to the *Cyprinidae* freshwater fish family (Spence, Gerlach, Lawrence, & Smith, 2008).

These fish are native of South Asia and are distributed along India, Bangladesh, Nepal, Myanmar, and Pakistan (Fig. 5) (Lawrence, 2007; Scholz et al., 2008). They inhabit rivers, canals, ditches and lakes (Froese & Capuli, 2011; Spence et al., 2008).

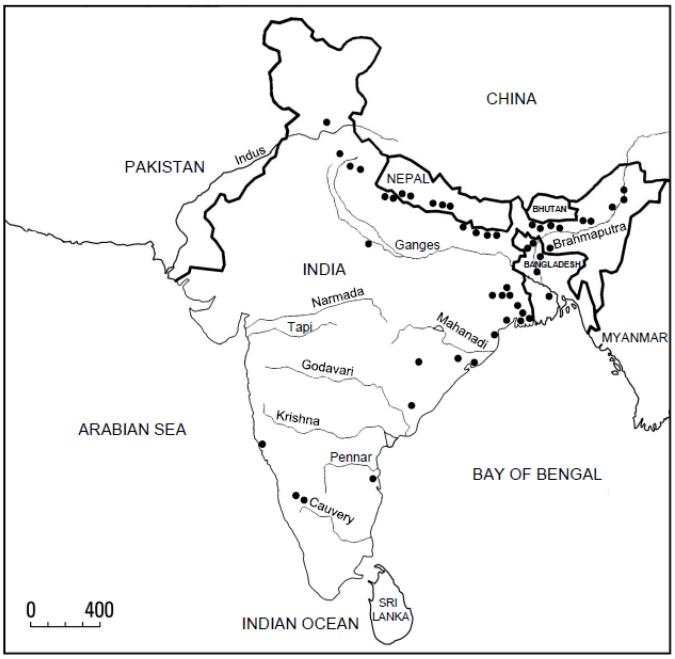

Figure 5- Zebrafish distribution marked with black dots (Spence et al., 2008)

The zebrafish have a small size (maximum is 4 cm), its body is fusiform and laterally flattened (Gómez-Laplaza & Gerlai, 2010; Spence et al., 2008). This species is characterized by 5-7 dark blue longitudinal lines from operculum into the caudal fin and anal fin striped (Froese & Capuli, 2011; Spence et al., 2008).

The females have more rounded body than males and although both have similar coloration, the males have an anal fin more yellow (and larger) than females (Figure 6) (Spence et al., 2008). They have an external fertilization, where in every spawn, each female can produce hundred eggs that are after fertilized by sperm released in water by males (Scholz et al., 2008; Spence et al., 2008).

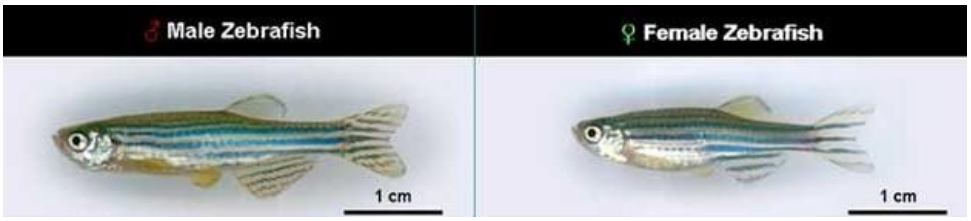

Figure 6 – Male and Female zebrafish (Lab, 2009)

The eggs are demersal, transparent and have a rapid development, being all the body formed at 24 hours post fertilization (hpf). The hatching occurs between 48 and 72 hpf and larvae reach maturity in 3-4months (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Scholz et al., 2008; Spence et al., 2008).

1.5.1. Zebrafish as a model test

Over the last decades, zebrafish has been widely used as a model test organism. Indeed, it is a powerful model being used in several areas of research, such as developmental biology (de Esch, Slieker, Wolterbeek, Woutersen, & de Groot, 2012), genetic and neurobiology (Sullivan & Kim, 2008), host-microbiota interactions (Roeselers et al., 2011b), learning and memory (Gómez-Laplaza & Gerlai, 2010), biomedical and human/animal disease (Giorgini et al., 2010) and (eco)toxicology (Scholz et al., 2008)

Actually, exceptional characteristics have led to its attraction. Firstly, their small size allow numerous organisms to be kept easily in a laboratory, it has a high reproductive rate, having each female hundred eggs in one spawn and rapid development (Chao et al.,

2010; Spence et al., 2008). In addition, both fertilization and development are external, being the eggs and larvae transparent which allows the monitoring of organs and mutant/transgenic lines development, the real-time observation of host-pathogen interaction and the response of immune system (Chao et al., 2010; de Esch et al., 2012). The adults are similar to mammals in relation of immune system response (Chao et al., 2010) and organs (Guo, 2009).

Moreover, its genome is completely sequenced and share about 60-80% of homolog genes with humans (de Esch et al., 2012; Howe et al., 2013; Meijer & Spaink, 2011). It is one of the best described organisms in developmental biology (Kimmel et al.) and have well described and broad molecular tools (de Esch et al., 2012).

1.6. Molecular Tools

Since the 30's, with the origin of molecular biology, the life sciences have suffering a revolution (Thakur et al., 2008). Indeed, new molecular techniques brought new areas of investigation and re-arranged others (Burton, 1996).

The base of these tools are the macromolecules such as nucleic acids and progressed in two areas, the PCR-based techniques and non-PCR-based techniques, being, nowadays, the first one broadly used in studies relating the diversity of microbial communities (Dorigo, Volatier, & Humbert, 2005; R. B. Gasser, 2006).

These tools are very sensitive, specific, reliable and faster, which made them widely used in microbiology (Giraffa & Neviani, 2001; Justé, Thomma, & Lievens, 2008), parasitology (R. B. Gasser, 2006) and marine ecology (Burton, 1996). Actually, the traditional culture methods are not able to show/understand all the complexity of the microbial communities, once it is estimated that less than 1% of bacteria in the environment are cultivable (Amann & Ludwig, 2000; Dorigo et al., 2005; Fontana, Vignolo, & Cocconcelli, 2005). Moreover, it solved problems related with time

consumption in traditional methods with slow growing bacteria, and demanding cultured organisms (Drancourt et al., 2000).

1.6.1. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was discovered by Mullis in 1983 (Mullis, 1990). The main principle of PCR is the amplification of a specific DNA fragment from a complex DNA sample (Burton, 1996; Gachet, Martin, Vigneau, & Meyer, 1998). This technique is based on the properties of double strands of the DNA, consisting in three steps: denaturing, annealing and extension (Fig. 7) (Gachet et al., 1998; R. B. Gasser, 2006; Tang & Stratton, 2012).

In denaturation step, the two strands of the DNA are separated by the increasing of temperature to 93/95ºC resulting in two single strands of DNA. This permits to endonucleases to cut specific regions of the DNA strand (Gachet et al., 1998; Tang & Stratton, 2012).

Later, the temperature is reduced to 50/60ºC which permits the primers to contact with the complementary target sequence. The primers are small artificial oligonucleotides (20 – 30 nucleotids), designed to recognize specific sequences of the DNA target. These annealing of the primers with the DNA will allow the DNA polymerase to bind and initiate the DNA synthesis (Gachet et al., 1998; R. B. Gasser, 2006; Tang & Stratton, 2012).

The extension step occurs at 72ºC, where the DNA polymerase synthetize the new strand (Gachet et al., 1998; R. B. Gasser, 2006; Tang & Stratton, 2012).

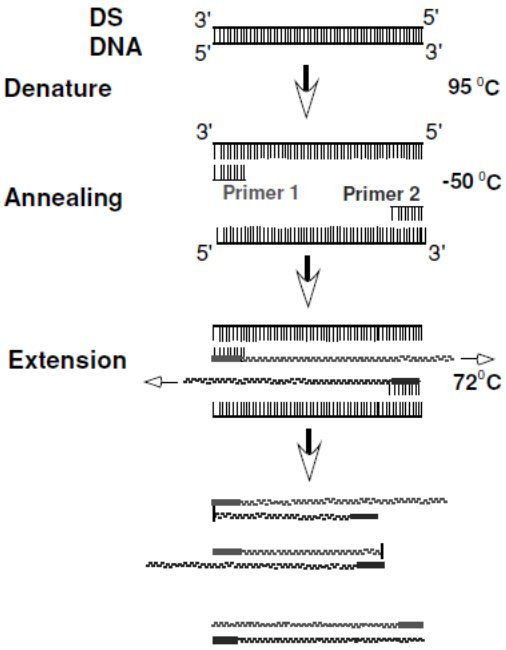

Figure 7 – PCR technique scheme (DS DNA= Double Strand DNA) (Tang & Stratton, 2012)

After each cycling, the double strand of DNA is doubled in two new double strands, which means that in a standard PCR with 20 to 50 cycles, millions of DNA strands could be obtainable for analysis (Gachet et al., 1998; R. B. Gasser, 2006; Mock, Richardson, & Wolf).

The primer target is the 16S ribosomal DNA, which is widely distributed among bacteria. The gene sequences of 16S rDNA is great conserved within the same species and different between species (Drancourt et al., 2000; Woo, Lau, Teng, Tse, & Yuen, 2008). This difference in gene composition will allow the separation of fragment and results theoretically in a band pattern characteristic of the bacterial population, their fingerprinting (Fontana et al., 2005).

Nested PCR

The Nested PCR consists in the amplification of the product of the fist PCR with a second one (Fig. 8) (Bayley and Scott's, 2007; Tang & Stratton, 2012).

In the first PCR is amplified a specific sequence of DNA target, next, in the second PCR is amplified a shorter sequence inside of the first amplicon (Bayley and Scott's, 2007; Tang & Stratton, 2012).

This technique increases the sensitivity and specificity of the reaction (Bayley and Scott's, 2007; Tang & Stratton, 2012).

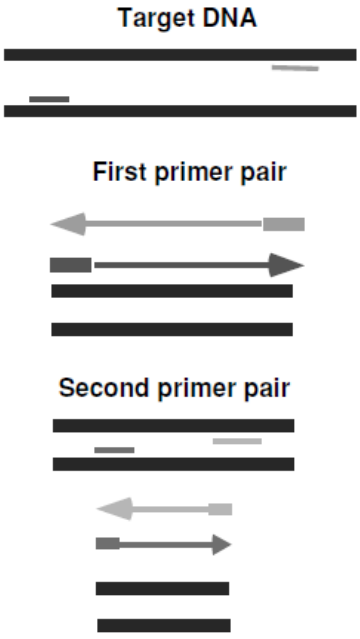

Figure 8 – Nested PCR scheme (Tang & Stratton, 2012)

1.6.2. Electrophoresis

The electrophoresis technique consists on the migration of charged molecules through a matrix gel (Bayley and Scott's, 2007). The molecules, usually DNA or RNA, are charged negatively and when exposed to an electrical field, are forced to migrate from negative pole to the positive one (Bayley and Scott's, 2007; T. M. d. S. Oliveira, 2010). Due to their different sizes, the fragments will migrate differently over the gel porous, being the smaller/lighter fragments on the bottom of the gel (Fig. 9) (Gachet et al., 1998).

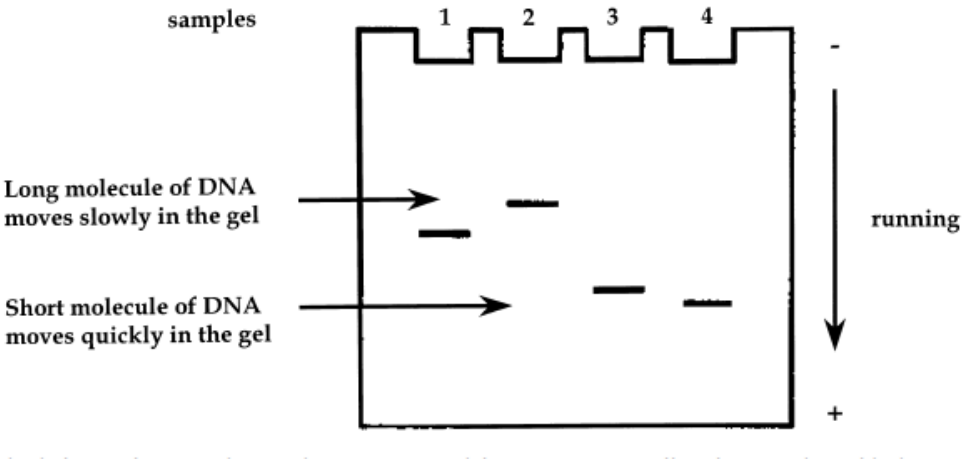

Figure 9- Schematic electrophoresis gel (Gachet et al., 1998)

The molecules are stained with a fluorescent dye, that binds DNA in order to the band pattern formed may be visualized under UV light (Bayley and Scott's, 2007; Gachet et al., 1998).

The electrophoresis technique is used after the PCR to determine/analyze if the previously technique worked and it was obtained the correct PCR product (Bayley and Scott's, 2007; Gachet et al., 1998).

1.6.3. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis is a technique based on an electrophoresis made on an acrylamide gel with a linear denaturing gradient (Gillan, 2004; Justé et al., 2008). The small DNA fragments are obtained by PCR and have the same size but different composition. These characteristic allows the physical separations by their melting properties, which depend on nucleotide sequence and GC content (Boutte, Grubisic, Balthasart, & Wilmotte, 2006; R. Gasser, Nansen, & Guldberg, 1996).

This technique is very used to get inter and intraspecific differentiation. Besides, the numbers of bands and their intensity can give an estimation of the community diversity and abundance (Dorigo et al., 2005; Manzano, Cocolin, Longo, & Comi, 2004).

1.7. Objectives

With the increasing of UV radiation reaching the earth's surface, and a variety of pollutants threatening the aquatic ecosystems, there is a need to understand how these stressors interact and what effects can be produced on organisms' survival and in their natural microflora, which plays an important role in animals' health.

In order to answer this question, the aim of this work is to evaluate the effects of combined toxicity of UV radiation and three xenobiotics on zebrafish embryos.

To achieve the main objective, the work was divided into the following steps:

- Evaluation of combined effects of UV radiation and xenobiotics triclosan, potassium dichromate and prochloraz, on zebrafish embryos;
- Characterization of zebrafish embryos natural bacterial communities at 24, 48 72 and 96 hours post fertilization;
- Characterization of zebrafish embryos natural bacterial communities in combined exposure of UV radiation and xenobiotics triclosan, potassium dichromate and prochloraz at 48 hours post fertilization.

1.8. Structure of the thesis

The current thesis is structured in the next chapters:

- Chapter 1: Introduction;
- Chapter 2: Interactive effects of Ultraviolet radiation and xenobiotic compounds in zebrafish embryos.
- Chapter 3: Combined effects of Ultraviolet radiation and xenobiotics on zebrafish embryos – changes in bacterial communities.
- Chapter 4: Conclusion.

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Chapter 2

INTERACTIVE EFFECTS OF ULTRAVIOLET RADIATION AND XENOBIOTIC COMPOUNDS IN ZEBRAFISH EMBRYOS

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Abstract

Climate change is an imperative problem compromising human and nature well-being. At ecosystems level, environmental parameters such as temperature, pH, dissolved oxygen concentration and intensity of UV radiation have an important role on the efficiency of organisms' physiological and behavioural performances and consequently on the capacity of response to contaminants. Insignificant alterations of these parameters may compromise this response. Understanding the combinatory effects of chemicals and environmental parameters is absolutely necessary for an adequate prediction of risk in aquatic environments. According with this scenario, this work aims at studying the combined toxicity of UV radiation and three xenobiotics, namely the biocide triclosan (TCS), the metal chromium (as potassium dichromate, PD) and the fungicide prochloraz (PCZ). To achieve this goal Zebrafish (Danio rerio) embryos (3 hpf) were exposed to several concentrations of each chemical combined with 5 different UV intensities for 8 hours. After this period normal light was used until the end of the test (96h). Embryos were daily inspected for mortality, developmental anomalies and development delay. Results showed different response patterns regarding the stresses levels and day of exposure. The combination of UV and TCS indicated a dose ratio deviation where synergism is observed when UV radiation is the dominant stressor. The combination of UV and PD presented dose level dependency at day 3 indicating antagonism at low stress levels. For day 4, dose ratio showed to be the best response and, in this case a synergism occurred in higher PD

concentrations. Finally, UV radiation combined with PCZ indicated a dose ratio at day 3 and dose level at day 4 of exposure, suggesting a synergistic response when PCZ is the dominant stressor in the combination. The obtained results in this study highlighted to the importance of including combined exposure scenarios to a best prediction of the risk.

Keywords: UV radiation, xenobiotics, *Danio rerio*, combined effects

1. Introduction

In the last years, is becoming apparent an overall warming trend. Indeed, multiple measurements made in the last 30 years, showed an increase of all wavelengths, having the ultraviolet radiation (UVR) from 200-300 nm increased more. As consequence UV radiation has been arriving surface of the earth with more intensity (Ribeiro, Ferreira, Ferreira, Soares, & Loureiro, 2011; Rowland, 2006). UVR can be divided in three categories: UV-A (315- 400), UV-B (280- 315 nm) and UV-C (100-280 nm) (Dong, Svoboda, Tiersch, & Todd Monroe, 2007). The UV-B is the most dangerous radiation for wild life as it can directly react with proteins and damage the DNA forming pyrimidine dimers, photoadducts and DNA-protein cross-links. On the other hand, UV-A is low absorbed by biomolecules, but can originate reactive oxygen species (ROS) such as single oxygen (Dahms & Lee, 2009). UV-C radiation is mostly absorbed by stratospheric ozone (Lars Behrensdt, 2010).

The main effect of UVR is the generation of ROS and subsequent radical formation that will damage cellular macromolecules (Lars Behrensdt, 2010). In the aquatic environment UVR causes severe effects on fish early development such as lesions in brain and retina development (Lars Behrensdt, 2010), decrease in the growth and development rates (Dahms & Lee, 2009). Literature has shown that UVR in adult fishes is immunosuppressive (Häkkinen et al., 2002; Sayed Ael, Ibrahim, Mekkawy, & Mahmoud, 2007). To face effects of UVR, organisms developed mechanisms of photoprotection, such

as screening pigments like melanin, photorepairing systems like photolyase, excision repairs and avoidance behavior (Hakkinen et al., 2002).

In a global environment increasingly disturbed by anthropogenic activities, organisms are very often subjected to other stressors as metals, pesticides among others. In natural ecosystems both aquatic and terrestrial species are frequently exposed to a combination of pollutants and fluctuations of abiotic conditions and possible interactions that between them can occur. (Barata, Baird, Nogueira, Agra, & Soares, 2007; Ferreira, Serra, Soares, & Loureiro, 2010; Holmstrup et al., 2010). The study of this theme is really important; the way that the interaction between chemicals and environmental factors occurs, the effects that they can promote in organisms can alert us to their ecological impact. Will these combinations promote a higher toxicity than alone? Will be benefit to organisms? Or their toxicity will not be affected by these combinations?

The purpose of this study was evaluate the effects of ultraviolet radiation combined with three chemicals namely triclosan (TCS), prochloraz (PCZ) and the metal chromium (as potassium dichromate, PD) on the development of zebrafish (*Danio rerio*) embryos. The zebrafish embryo was chosen to perform this study because the eggs and post hatched larvae are transparent, therefore we can screen the entire organogenesis delays and anomalies in specific organs and their development is really fast (Inês Domingues, 2010).

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a common bactericide very used in oral care products (Irgacare MP), acrylic products (Biofresh©), plastic materials (Microban©) and products for skin applications (Dann & Hontela, 2011; Rhaul Oliveira, 2009). Because of all these applications, this compound has been increasingly used (Sankoda et al., 2010). Despite being deposed with domestic wastewater arriving later to treatment station, there are studies that indicate the presence of Triclosan on surface waters (Bedoux, Roig, Thomas, Dupont, & Le Bot, 2012; Benotti et al., 2009; Bester, 2003; Kolpin et al., 2002; Lindstrom et al., 2002; Loraine & Pettigrove, 2006), in concentrations ranging from 0.001 µg L^{-1} to 2.3 µg L^{-1} (Brausch & Rand, 2011). Recently, many studies have also investigated TCS toxicity in aquatic organisms such as algae, mollusks, crustaceans and fish (J. W. Kim et al., 2009; Orvos et al., 2002; Oliveira, Domingues, 2009; Riva, Cristoni, & Binelli, 2012; Tatarazako, Ishibashi, Teshima, Kishi, & Arizono, 2004).

Potassium dichromate ($K_2Cr_2O_7$) was used as the source of chromium (VI). Actually, chromium can occur from natural processes, but hexavalent chromium unlike others forms is not common in nature, rather being used in anthropogenic activity (Barnhart, 1997; Goodale et al., 2008). Chromium is used on a large scale in industry, including metallurgical, production of paints and pigments, tanning, wood preservation among others (Zayed & Terry, 2003). Fishes are more susceptible to toxic impact of hexavalent form of chromium than other aquatic organisms. It can easily induce oxidative stress (Begum, Venkateswara Rao, & Srikanth, 2006; Lushchak, Kubrak, Nykorak, Storey, & Lushchak, 2008; Roberts & Oris, 2004; Velma & Tchounwou, 2011), affect the immune system (Khangarot, Rathore, & Tripathi, 1999; Vutukuru, 2003) and can be genotoxic and cytotoxic to fish cells (Goodale et al., 2008; Velma & Tchounwou, 2010).

Prochloraz has been used since the early 1970s in agriculture. It is a broad-spectrum fungicide, employed in Europe, South America, Asia and Australia (Sanchez, Piccini, & Porcher, 2007). In freshwater ecosystems, PCZ can generate adverse effects to aquatic organisms (Bjergager, Hanson, Solomon, & Cedergreen, 2012; Brande-Lavridsen, Christensen-Dalsgaard, & Korsgaard, 2008; Cedergreen, Kamper, & Streibig, 2006; Le Gac, Thomas, Mourot, & Loir, 2001). Moreover studies have proved that PCZ can be very toxic to fish (Domingues et al., 2011; Le Gac et al., 2001; Sanchez et al., 2007; Sturm, Cravedi, Perdu, Baradat, & Segner, 2001) According to this studies PCZ act as an inhibitor of ergosterol biosynthesis (EBI), which means that exert their effect based on the inhibition of cytochrome P450-dependent step in ergosol synthesis (Domingues et al., 2011; Sturm et al., 2001). This compound can also produce dramatic changes in hepatic and intestinal microsomal mono-oxygenases (Rivière, Bach, & Grolleau, 1985).

In order to predict the joint effects of UV radiation and the three chemicals on zebrafish embryos, the two reference conceptual models of Concentration Addiction (CA) and Independent Action (IA) were adopted in this study. The CA concept is applied when each of the components in the mixture act in the same way and presuppose that the toxicity of the mixture is a summation of the doses of the individual compounds. The IA concept assumes that the modes of action differ among the components in the mixture and the effect of each compound will have is a matter of probability (Groten, 2000). However in real scenarios sometimes the interaction between stressors may modify the magnitude and

the nature of toxic effect. There are already reported cases where combinations of stressors interacted with one another producing different behavior, diverging from the pattern such as those producing more severe (synergism), or less severe effect (antagonism). Furthermore the responses might follow a dose level relationship where the toxicity depends of the dose level of the stressor (i.e different effects at low and high dose levels) and a dose ratio relationship that is observed when the toxicity of the mixture or combination is principally caused by one of the components (Jonker, Svendsen, Bedaux, Bongers, & Kammenga, 2005).

2. Materials and Methods

Test organisms

The Zebrafish (Danio rerio) facility established at the Biology department from University of Aveiro (Portugal) provided all organisms used in this study. The organisms are maintained in carbon-filtered water at 26 ±1º C under a photoperiod cycle 16:8h light/dark. Conductivity is maintained on 750 ±50 µs, pH at 7.5 ±0.5 and dissolved oxygen at 95% saturation.

The assay was based on OECD guideline on Fish Embryo Toxicity Test (OECD 2013). 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. In combined test of UVR with TCS and PD were used 250 eggs while in the combined test of UVR with PCZ were used 300 eggs which were distributed in petri dishes with different toxicant concentrations of xenobiotic in test and placed under UV light.

Chemicals

Zebrafish embryos were exposed to several concentrations of triclosan (TCS), potassium dichromate (PD) and prochloraz (PCZ) combined with UV radiation. TCS (Irgasan, 5-chloro-2-(2,4-dichlorophenoxy)phenol) was purchased from Sigma- Aldrich. Stock solution was prepared by dissolving TCS in acetone and the test solutions were obtained by diluting the stock in water. Nominal concentrations of triclosan ranged from 0.1 mg/L to 0.7 mg/L. PD (K2Cr2O7) was used as a source of chromium (VI). The different solutions of 100 to 500 mg/L, were prepared diluting a Stock obtained by dissolution of potassium dichromate in water. PCZ (1-[N-propyl-N-2-(2,4,6-trichlorophenoxy) ethylcarbamoyl] imidazole I: C15H16Cl3N3O2) PESTANAL ®, (CAS Number: 67747-09-05) was acquires from Sigma-Aldrich Biotechnology LP. In order to obtain stock solution, PCZ were dissolved in acetone and test solutions ranging from 0.6 to 8.9 mg/L were obtained by diluting stock in water.

Ultraviolet Radiation

Ultraviolet radiation was provided by UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312 nm). All exposure to radiation was performed in a controlled temperature room (26 ±1º C). A UV-C radiation filter previously UV irradiated for 12h was used in order to minimize the differences in UV radiation intensity passing through the UV lamp. The UV dose ($J.m^{-2}$) was obtained by taking into account the time of exposure and was calculated as: UV dose ($J\ m^{-2}$)= UV intensity ($mW\ m^{-2}$) × time of exposure (s)/10^3. Intensities of issued UV radiation were measured with a spectro-radiometer placed at surface water level and connected to a monochromator that provided information on energy per nanometer. Intensities were measured before and after each exposure. Spectral irradiance was obtained by the BenWin+ software (Benthan Instruments, Reading, UK). A preliminary test, only with UV radiation, was made in order to adapt the different intensities to be used in the combined experiments.

Combination of chemicals and UV radiation

The embryos were exposed to four different intensities of UV radiation combined with five concentrations of TCS, PD and six concentrations of PCZ during eight hours. The intensities were achieved by exposing the embryos at different distance from the UV lamp. Table 5 present the intensities and the equivalent UV doses for each combined exposure. After the eight hours under UV light the embryos were transferred to 24 well plates, continuing exposed to the chemicals, but now with natural light. Mortality, hatching, pigmentation, development delay, absorption of yolk sac, formation of edema and malformations of tail were observed daily, for 96h.

Table 5 - UV intensities (mW m^{-2}) and correspondent doses for each combined exposure.

Combination	UV intensity (mW m^{-2})	UV dose (kJ m^{-2})
UV x TCS	0	0
	365.72	10.53
	637.07	18.35
	1241.25	35.75
	1886.28	54.32
UV x PD	0	0
	195.84	5.64
	463.41	13.35
	1181.10	34.02
	2468.30	71.09
UV x PCZ	0	0
	217.20	6.26
	480.90	13.85
	1005.94	28.97
	2355.07	67.83

Chemicals Analysis

In order to verify the nominal concentrations of the chemicals used and effects of UV light on their possible degradation, analysis of TCS, PD and PCZ in the water samples were conducted. The water samples were collected at time 0h, 24h, 72h and 96h in the

lowest and highest concentration of each chemical and in the lowest and highest UV intensity. Determinations of the PCZ and TCS concentration in our samples were performed at Laboratory of Environmental Chemistry and Biochemistry, University of South Bohemia in Czech Budejovice. Prochloraz and triclosan in the water samples were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The protocol was adapted from Grabic, Fick et al. (2012). An external calibration was used. For the LC-MS/MS injections, water samples with the highest concentration of prochloraz compound were diluted prior to analysis in tap water (1:10). These analyses were performed with a TSQ Ultra MS/MS (Thermo Fisher Scientific, San Jose, CA, USA) coupled with Accela 1250 pump (Thermo Fisher Scientific, San Jose, CA, USA) and a HTS-XT autosampler (CTC Analytics AG, Zwingen, Switzerland). HESI II ion source was used for the ionization of prochloraz, APCI coupled with APPI ion source – for the ionization of triclosan. The average relative recovery for prochloraz and triclosan in water were 96 % with RSD 15 % and 88 % with RSD 17 %, respectively. The limits of quantification (LOQ) for prochloraz and triclosan in water samples were 0.5 and 0.3 ng mL^{-1}, respectively. Determinations of chromium were performed at the Laboratório Central de Análises (LCA), University of Aveiro. Chromium in the water samples was determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES), methodology based on ISO 11885:2003. The limit of quantification for chromium in water samples was 0.1 µg L^{-1}.

Data analysis

The prediction of toxicity of combined exposure to UV radiation and xenobiotics was based on the conceptual model of Independent Action (IA) which admits that both components in the combination have dissimilar modes of action. Under a combination, the toxicity behaviors of a compound can deviate from the conceptual model, being more or less toxic than expected. Thus, the deviations from IA to antagonism/synergism, dose level and dose ratio dependent response were analyzed with the MIXTOX tool (Jonker, Svendsen et al. 2005) which allows to test the most significant adjustment of the data and achieve the best description of the biological response pattern.

3. Results

Chemicals Concentrations

Figures S1, S2 and S3 (See Supplementary data) present the nominal concentrations of TCS, PD and PCZ and its respective measured concentrations. The concentration of TCS measured in water samples in time 0 varied 32% of the intended concentrations corresponding to 0.47 mg/L. The degradation of TCS increased with time and UV exposure reaching more than 99% at 96h of exposure.

UV radiation seems to have no effect on PD and PCZ. The average concentrations of PCZ measured in water samples varied within 26% of the intended concentrations and corresponded to 0.84±0.17 and 13.44±2.13 mg/L, which in turn corresponds to the nominal concentrations of 0.6 and 8.9 mg/L. In case of PD, the average concentration measured in water samples varied within 20.5% of the intended concentrations and corresponded to 116.62±21.09 and 545.82±76 mg/L, which in turn corresponds to the nominal concentrations of 100 and 500 mg/L.

Combined toxicity

The development of zebrafish embryos (96h) was evaluated after exposure to increasing UV radiation and concentrations of PD, PCZ and TCS. In order to predict the toxicity of these combinations the MixTox model was assessed to generate the optimal description of the biological response of zebrafish embryos to the combination of these natural and chemical stressors (table 6). The conceptual model Independent Action (IA) was essentially the best to fit our data set, considering the dissimilarity on stressors mode of action on the organism. All the parameters obtained with the MixTox model are presented in table 7.

Table 6 - Interpretation of parameters that define the functional form of deviation pattern from concentration addiction (CA) and independent action (AI) (adapted from Jonker at al. 2005)

Deviation Pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (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 positive b value indicate antagonism	b_i**<0:** synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	**a>0:** antagonism low dose level and synergism high dose level	b_{DL}**>1:** change at lower EC50 level	b_{DL}**>2:** change at lower EC50 level
		b_{DL}**=1:** change at EC50 level	b_{DL}**=2:** change at EC50 level
		0<b_{DL}<**1:** change at higher EC50 level	**1**<b_{DL}<**2:** change at higher EC50 level
	a<0: synergism low dose level and antagonism high dose level	b_{DL}<**0:** No change but the magnitude of S/A is DL dependent	b_{DL}<**1:** No change but the magnitude of S/A is effect level dependent

Table 7 - Summary of MixTox analyses for the combination of UV radiation and triclosan, potassium dichromate or prochloraz to zebrafish survival.

	UV x TCS	UV x PD		UV x PCZ	
	Day 2	**Day 3**	**Day 4**	**Day 3**	**Day 4**
R^2	0.833	0.819	0.897	0.757	0.853
SS	29.63	31.023	16.815	61.352	40.616
A	12.95	0.044	-6.368	-13.75	64.77
B	-14.33	-167.87	30.48	22.25	2.025
p-value	8.65E-06	4.3E-05	9.22E-08	0.0007	4.57E-05
LC50	UV = 14.23 TCS = 0.49	UV = 12.40 PD = 232.17	UV = 11.13 PD = 176.70	UV = 20.82 PCZ = 7.62	UV = 29.29 PCZ = 6.31
Slope	UV = 2.93 TCS = 6.26	UV = 1.59 PD = 12.87	UV = 3.70 PD = 13.04	UV = 2.05 PCZ = 7.80	UV =0.20 PCZ =365.08
Max	0.98	0.98	0.83	0.98	0.98
Deviation Pattern	Dose-ratio dependent	Dose-level dependent	Dose-ratio dependent	Dose-ratio dependent	Dose-level dependent

UVR and TCS

The toxicity of the combination of UV and TCS on the survival of zebrafish embryos fitted to the IA model, but when changing the functions to assess deviations a dose ratio deviation showed to be the best description (R^2=0.83, SS=29.62 p value), based on the maximum likelihood test. This response pattern means that antagonism is expected, however, the interpretation of the parameters a and b (a = 12.95, b = -14.33) indicated that when UV radiation is the dominant stressor in the combination a synergism pattern may occur. From figure 10 it can be observed that as the UV radiation increases, the survival of zebrafish embryos decreases.

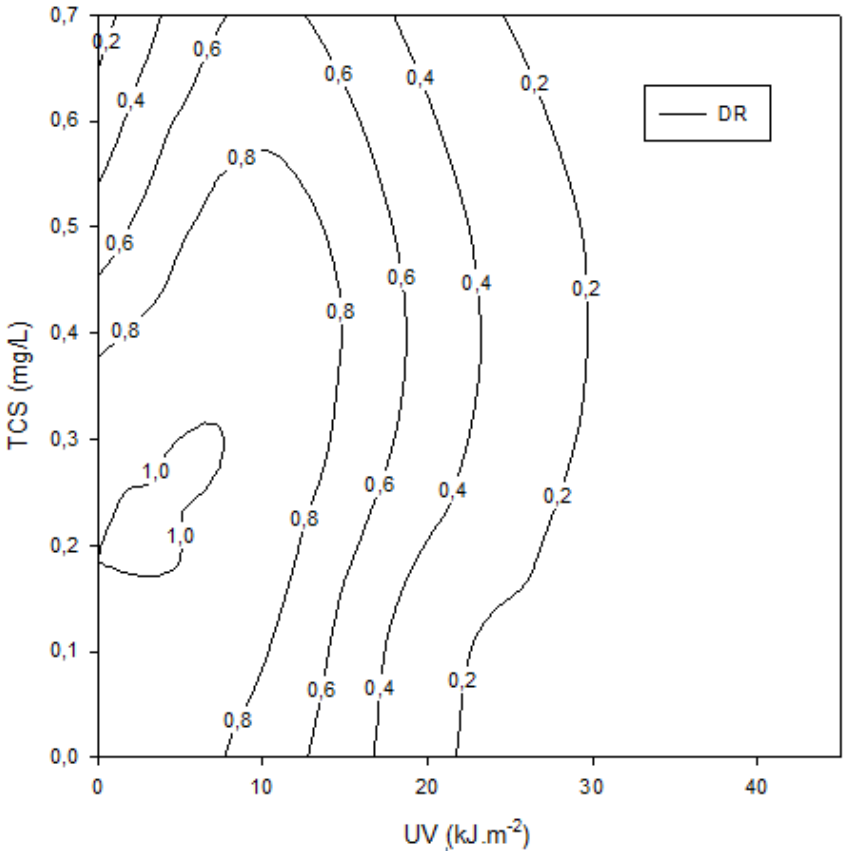

Figure 10 - Dose response relationship of survival rates of zebrafish embryos at day 2 exposed to the combination of UV radiation and TCS, showing a dose-ratio deviation from the IA conceptual model.

UVR and PD

For the combination of UVR and PD, two distinct deviation patterns from IA model were observed: Dose Level (DL) at day 3 and Dose Ratio (DR) at day 4. At day 3 the survival of zebrafish embryos was adjusted by the IA model (SS=51.13, R^2=0.70, p = 3.90 x 10^{-25}), but the addition of parameter a to the regression equation increased the R^2 value (0.78) and decreased the SS (36.78) value suggesting an antagonistic pattern for the combination. Nevertheless, the addition of parameter b to the equation showed that a dose level deviation pattern (SS=31.02, R^2=0.81, p = 4.30 x 10^{-5}) was the most effective to describe our data, supported by the maximum likelihood test. Moreover, the positive value for parameter a (a = 0.043) indicated antagonism at low stress levels, and synergism at high dose level. Accordingly, the change from antagonism to synergism (predicted by the model) will occur at higher doses than the ones used (Fig. 11 A). On the other hand, at day 4 a dose ration deviation pattern (SS=16.81, R^2=0.89, p = 9.22 x 10^{-8}) showed to be the best characterization for the data set. The interpretation of parameter a (a = -6.36) and b (b = 30.48) suggests that when PD is the dominant stressor in combination, i.e., at high concentrations of PD and low doses of UV radiation, synergism is likely to occur. Conversely, antagonism occurs when the ultraviolet radiation is the dominant stressor in the combination, i.e. at elevated UV doses and low concentrations of PD, the number of survivors embryos was higher than the one predicted by the model.

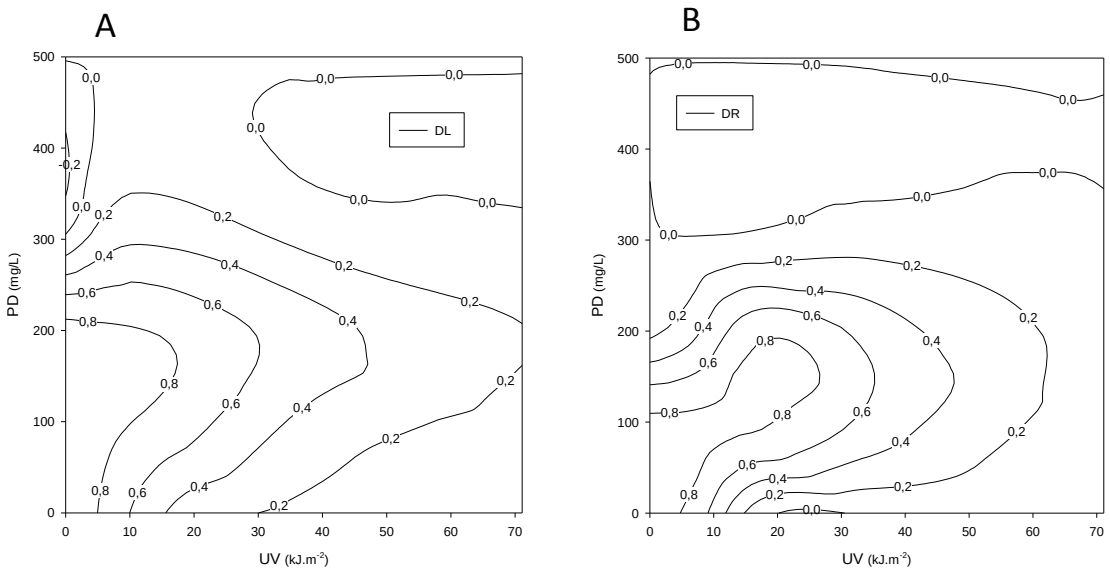

Figure 11 - Dose response relationship of survival rates of zebrafish embryos exposed to the combination of UV radiation and potassium dichromate, showing a dose-level dependency at day 3(A) and dose-ratio response at day 4 of exposure (B).

UVR and PCZ

The response to the combination of UV radiation and PCZ also showed different deviation patterns from IA regarding the day of exposure. At day 3 a Dose ratio dependency (SS=61.35, R^2=0.76, p = 7.4 x 10^{-4}) was detected by changing the functions, namely parameters a and b, to assess deviations. The negative value of parameter a (a = -13.754) indicated synergism when PCZ is dominant in the combination i.e., low UV radiation intensities combined with high PCZ concentrations will result in a synergistic pattern (Fig. 12). Inversely, the positive value of parameter b (b = 22.2519) indicated antagonism when UV is dominant, i.e., high UV radiation combined with low PCZ will result in an antagonistic pattern (Fig. 12 A). Data from day 4 although fitted the IA model, showed a Dose level dependency when tested for possible deviation patterns (SS=40.61, R^2=0.85, p = 4.75 x 10^{-5}). The interpretation of positive parameters a and b (a = 64.7, b = 2.025) suggested an antagonistic behavior at low stress levels and synergism at high stress levels (Fig. 12 B). The b value (b = 2.025) indicated that the change from antagonism to synergism occurred at the LC 50 level (UV = 29.29 kJ m^{-2}, PCZ = 6.31 mg L^{-1}).

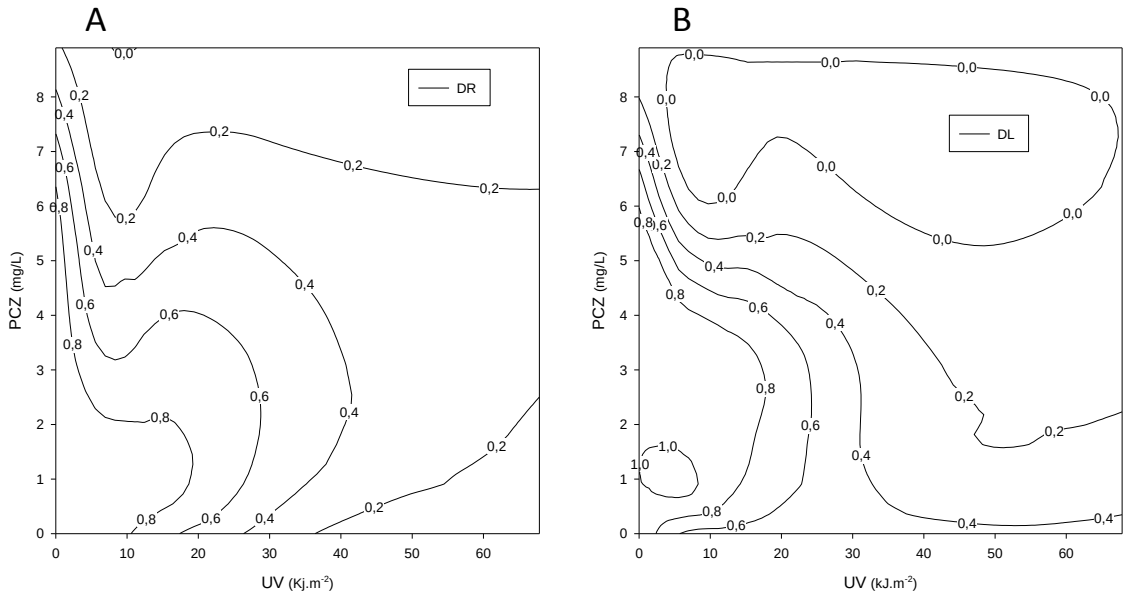

Figure 12 - Dose response relationship of survival rates of zebrafish embryos exposed to the combination of UV radiation and prochloraz, showing a dose-ratio at day 3 of exposure (A) and dose-level response at day 4 (B).

4. Discussion

In our work, we showed the interactive effect of UV radiation and toxicants in early life stages of zebrafish.

In chemicals analysis, the lower measured concentrations of TCS can be explained by the possible photodegradation caused by light exposure, especially UV light. Several authors have already demonstrated that TCS is easily and quickly degraded with sun and UV light exposure (Latch, Packer, Arnold, & McNeill, 2003; Latch et al., 2005; Mezcua et al., 2004; Sanchez-Prado, Llompart, Lores, Fernandez-Alvarez, et al., 2006; Singer, Müller, Tixier, & Pillonel, 2002). Sanchez-Prado, Llompart et al. (2006) found a half-life time for photodegradation of TCS of 4.8 min under UV light and 3.9 min under sunlight exposure. Although the phototransformation seems to be one of the main eliminations pathways of TCS in the aquatic environment, there is a special concern on the generation of highly toxic photoproducts. According to a number of studies, TCS degradation leads to

the formation of various photoproducts including monochlorophenol, 2,4-dichlorophenol (considered a priority pollutant), methyltriclosan, 2,8-DCDD (Latch et al., 2003; Latch et al., 2005; Mezcua et al., 2004), dichlorohydroxydiphenyl ether and monochlorohydroxydiphenyl ether (Sanchez-Prado, Llompart, Lores, Garcia-Jares, et al., 2006).

Interpretation of the modeled data for the combination of TCS and UV radiation indicated a dose ratio deviation from IA model. The analysis of parameter a suggested an antagonistic pattern mainly caused by the chemical stressor (TCS). However, parameter b indicated synergism at day 2 of exposure, when UV radiation was the dominant stressor in the combination. Therefore, it pointed out that at high doses of UV radiation and low concentrations of TCS, the survival of zebrafish embryos decreased. The antagonistic pattern observed can be explained by photomodification of TCS when exposed to light. Many studies, which investigated the environmental fate of TCS, found that this chemical is easily degraded by UV light exposure (Chen, Cao, & Song; Latch et al., 2003; Mezcua et al., 2004). According to (Sanchez-Prado, Llompart, Lores, Garcia-Jares, et al., 2006) TCS has a very fast half-life of 4.8 min under UV light and after 30 min of irradiation its response was lower than 7% of the initial response. Regarding our experimental design that includes a UV exposure of 8 hours, the TCS was almost all degraded reaching 99% of degradation even in the highest concentration used.

Conversely, it is of special concern the generation of highly toxic photoproducts as a function of photo-degradation of TCS. The degradation of TCS can lead to the formation of several photoproducts including monochlorophenol, 2,4-dichlorophenol, 2,8-DCDD, dichlorohydroxydiphenyl ether and monochlorohydroxydiphenyl ether, some of them considered to be of public health concern (Dann & Hontela, 2011; Latch et al., 2005). The generation of these photoproducts might elucidate the synergism pattern when UV is the dominant stressor in the combination, once the UV radiation can trigger the formation of very toxic TCS photoproducts. It has been shown that methyl-triclosan (degradation product of TCS) is also very toxic to aquatic species (Farré et al., 2008). Another study, which evaluated the combined toxicity of UV radiation and a carbamate insecticide (Carbaryl) to early life stages of amphibians, also found a synergistic pattern effect on mortality in high UV-B levels (Zaga, Little, Rabeni, & Ellersieck, 1998) which was

attributed to the photomodification of carbaryl in the water. Other studies investigating the combined toxicity of UV radiation and organic compounds to *Daphnia magna*, presented as well a synergistic response mainly caused by phototransformation (or photomodification) of chemicals compounds (Huovinen, Soimasuo, & Oikari, 2001; Kim, Park, & Choi, 2009; Nikkilä, Penttinen, & Kukkonen, 1999; Scrano et al., 2002).

A deeper understanding of TCS and UV interaction would only be reached by the study of the mode of action of the combined stressors using molecular analysis. Moreover, measuring TCS metabolites in the exposure medium would also be important as these compounds can also be contributing to the toxicity observed and can also be interacting with UV radiation.

On the other hand, the interaction of UV radiation and PD showed an antagonistic effect for low dose level at day 3. Actually, this effect was also perceived in collagen exposed to chromium (III) and UVR by Fathima et al. (2008). In their work, they observed an increasing of stability of collagen treated with chromium against UVR than those without treatment. Moreover, a study conducted by White & Jahnke (2004) showed the filtering effect of chromate removing UV-A and UV-C radiation. They proved that chromate can be more effective than acetate filter in the separation of UV-B radiation from UV-A and UV-C. The chromate filter was able to removing all UV-A radiation shorter than 340nm and most of radiation between 340-320nm, while acetate can only absorbed all UV-A radiation shorter than 380nm. At day 4, a dose ratio pattern was achieved, which means that for those mixtures where PD is dominant, a synergism is expected. This effect can be explained by the enhanced of carcinogenesis effect and oxidative stress of PD by UV radiation. In fact, (Uddin et al., 2007) demonstrated the UV-induced carcinogenesis effect in mice exposed to potassium dichromate, which increases through the duration of the experiment.

UV radiation and PCZ had a synergistic effect at day 3 on zebrafish early life stages when the chemical is dominant in the mixture. Other studies have already showed the synergistic effect in the combination of pesticides with other chemicals on *Vibrio fischerie*, *Lemna minor* and *Daphnia magna* (Cedergreen et al., 2006). Further, the combined effect of pesticides and UV radiation was also studied by (Beketov, Speranza, & Liess, 2011) and Ribeiro et al. (2011) on *Daphnia magna.* In both studies were observed negative effects of

the combination on reproductive rate and mortality. However, Beketov et al. (2011) also showed that the negative effect of the combination depends on the composition of the pesticide and their concentration, which is according with our results where an antagonism effect is expected when PCZ are in low concentration and UV radiation is dominant.

The response patterns observed in this study are possibly related to different processes. In the case of TCS, a photomodification seems to occur when UV radiation is the dominant stressor, probably generating photoproducts more toxic than TCS itself. UV has been shown repeatedly to cause degradation of TCS and also to generate high toxic photoproducts (Dann & Hontela, 2011; Latch et al., 2005). Another explanation can be a photosensitization phenomenon: higher UV radiation could increase the sensitivity of zebrafish embryos to TCS, causing synergism even in the lowest concentrations. In the case of PD and PCZ the toxicity seems to depend on the composition and concentrations of the pesticides. In the combination of UV-B and PD, the protective capacity of chromate may elucidate the antagonism in low stresses levels. On the other hand in the higher stresses levels, the observed synergistic effects are probably due to the combined effects of the two stressors on embryo physiology. Observed interactions between UV radiation and PCZ also arise from combined physiological stress to zebrafish embryos caused by both stressors. Nevertheless, further studies are necessary to better understand the mechanism behind theses interactions

5. Conclusion

In their natural environment, organisms are exposed to a multitude of biotic and abiotic stressors that can interact with each other producing synergistic or antagonistic effect. The results of the present study demonstrate that different interactive responses of combinations between UV radiation and chemical compounds may occur. These interactive effects, as have been shown, threat the aquatic biota, highlighting to the need to take in consideration environmental factors as additional stress in ecotoxicology studies. Our study also demonstrates that these interactive effects have to be carefully evaluated in order to predict safe concentrations of these chemicals in the environment to avoid under

or overestimation of their effects. Additionally, more studies need to be conducted to clarify the mechanism of interactions between UV radiation and chemical compounds.

Acknowledgements

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

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Supplementary data

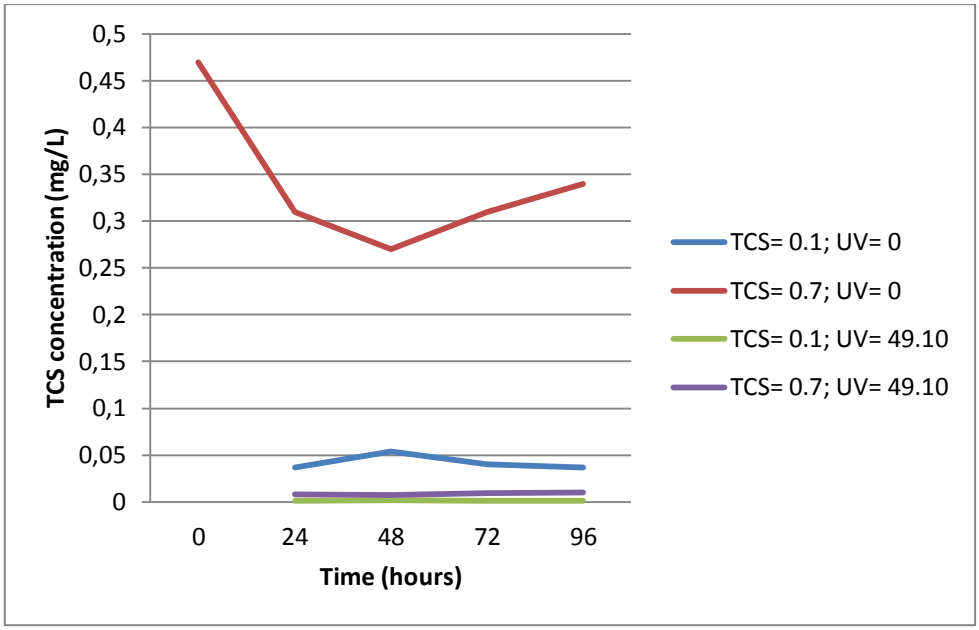

Figure S 1 - Decay curve of measured concentrations (mg/L) of triclosan under UV radiation (49.10 kJ/m2) or normal light during 96 hours.

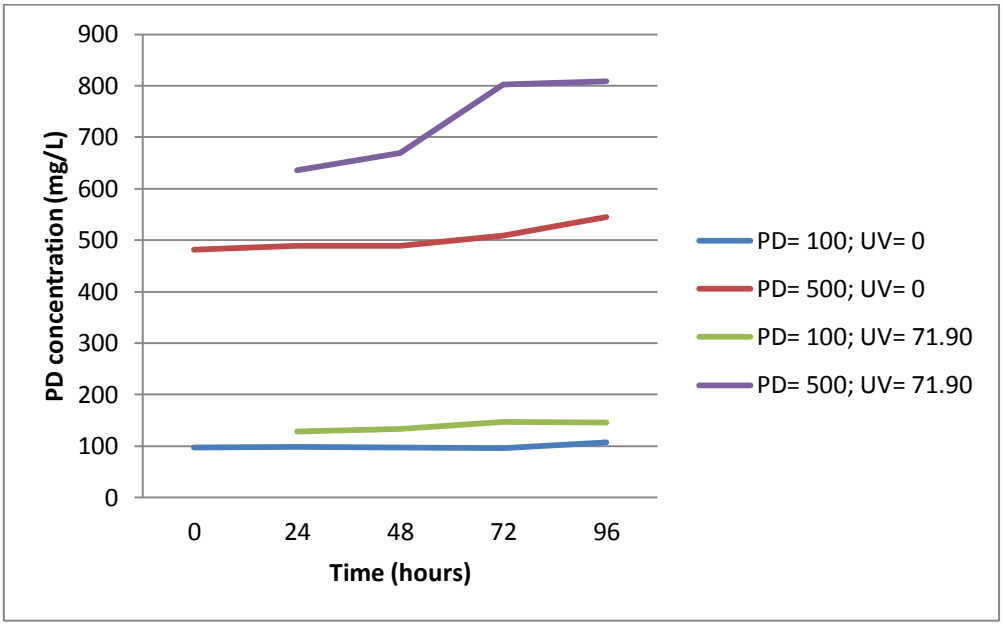

Figure S 2 - Measured concentrations (mg/L) of potassium dichromate under UV radiation (71.10 kJ/m^2) or normal light during 96hours.

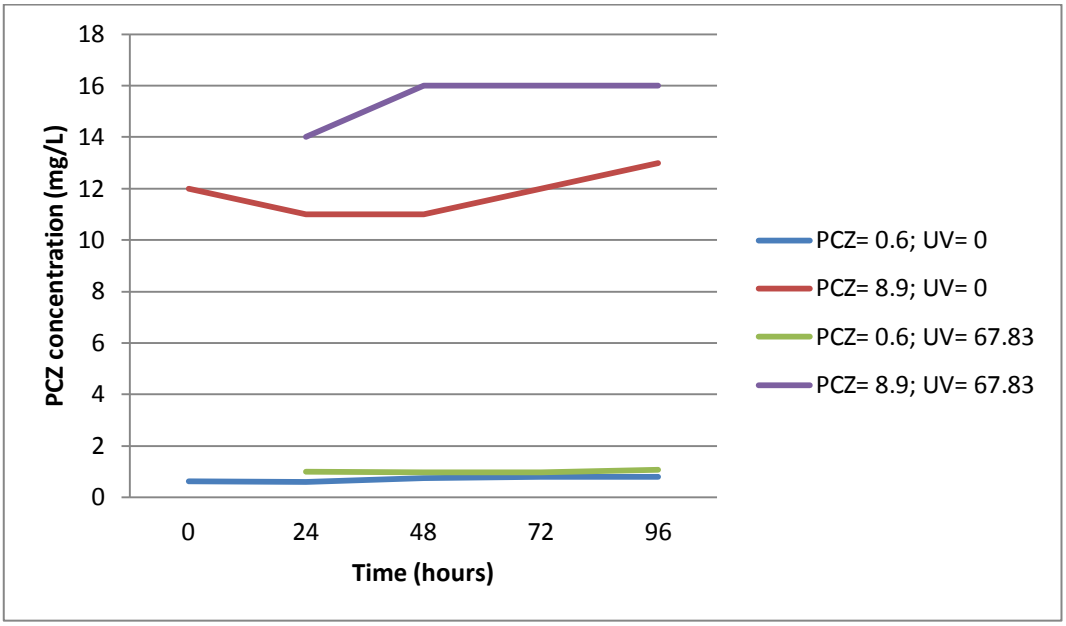

Figure S 3- Measured concentrations (mg/L) of prochloraz under UV radiation (67.83 kJ/m^2) or normal light during 96hours.

Chapter 3

COMBINED EFFECTS OF ULTRAVIOLET RADIATION AND XENOBIOTICS ON ZEBRAFISH EMBRYOS - CHANGES IN BACTERIAL COMMUNITIES

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Abstract

In their natural environment, organisms establish intimal associations with surrounding microbial communities, constituting their own microflora. Natural microflora plays an important role on host health, contributing to their immune system, being the "first line of defense" against pathogens, furnishing some nutrients that host could not access alone (e.g. vitamins, minerals and enzymes), conditioning behavioral patterns and weight regulation. In aquatic ecosystems some environmental parameters (e.g. Ultaviolet radiation, temperature, oxygen levels and pH) and/or pollutants can constitute stress factors weakening this "first line of defense" of the organism. There are now strong evidences suggesting that chemicals and environmental stressors can interact synergistically augmenting the expected toxicity for organisms; however the effects of these possible interactions have not been studied in organisms' natural microbial diversity. With the global climate changes, it is expected that environmental parameters such as UV radiation may increasingly act as co-stressors. Thus, this work aims at studying the effects of combined exposure to UV radiation and three xenobiotics (triclosan (TCS), potassium dichromate (PD) and prochloraz (PCZ)) on bacterial communities associated to zebrafish (Danio rerio) embryos. Zebrafish embryos were exposed to three different intensities of UV radiation combined with three different concentrations of each chemical for 8 hours. After this period embryos were only exposed to the chemical, and at 48 hours post fertilization the molecular analysis (DNA extraction, polymerase chain reaction (PCR) and

denaturing gradient gel electrophoresis (DGGE)) was performed. DGGE provides information about microbial community diversity and abundance through the analysis of the number of bands and their intensity. The UV radiation showed to have an effect on bacterial community structure however there was no differences in the diversity.

Combined test of UV dose with TCS revealed a change in band pattern with the shift of bacterial communities. Moreover, the combinative test of UV radiation with PD showed a significant reduces of bacterial communities at higher PD concentration. PCZ seems do not have a significant effect on bacteria communities. Natural communities are essential to the eggs health and survival could changes in bacterial communities' structure compromising eggs development.

Keywords: UV radiation, *Danio rerio*, xenobiotics, bacterial communities

1. Introduction

In the last years, climate changes have highlighted the importance of including environmental factors as potential additional stressors (Beketov, Speranza, & Liess, 2011) for aquatic organisms. Indeed, in natural environments, the fitness of organisms may be compromised not only by a multiplicity of pollutants derived mostly from anthropogenic activity, but also by abiotic components such as pH, UV radiation and dissolved oxygen concentrations. There are now strong evidences that these two types of factors can interact (Beketov et al., 2011; Tu et al., 2012) resulting in an increased environmental risk for aquatic ecosystems.

Some studies have already documented significant interactions between temperature, salinity and toxicants in grass shrimp (*Palaemonetes pugio*), in zooplankton, in rotifer (*Brachionus rotundiformis*) and in black tiger shrimp (*Penaeus monodon*) (Gama-Flores, Sarma, & Nandini, 2005; Howard & Hacker, 1990; Tu et al., 2012). The toxicity of metals and pesticides with high temperatures was also demonstrated in crayfish (*Orconectes immunis*) and earthworm (*Lumbricus terrestris*) (Khan et al., 2006; Khan et

al., 2007), in zebrafish (*Danio rerio*) (Hallare, Schirling, Luckenbach, Köhler, & Triebskorn, 2005; Scheil & Köhler, 2009) and daphnia (*Daphnia pulex*) (Boeckman & Bidwell, 2006).

Records from the last years showed an increase of all wavelengths of solar radiance, especially UV radiation (Lean, 2010; Lean & DeLand, 2012). Moreover, the interactive effect of UV radiation and chemicals was already revealed. Huovinen et al (2001) showed a synergistic effect between UV radiation and polycyclic aromatic hydrocarbons, Jung et al. (2008) also showed the interactive effect of UV radiation and sulfonamide antibiotics in *Daphnia magna*. A synergism between UV radiation and carbendazim was also observed by Ribeiro et al (2011), when the toxicity of combination was produced mainly by UV radiation. In addition, Kim et al. (2009) showed an increase of oxidative stress when *D.magna* were exposed to a different UV-B levels combined with and sulfathiazole and Nikillä et al. (1999) observed an enhanced toxicity of pyrene under UV-B radiation.

Furthermore, the interaction between UV radiation and chemical stressors might also have an effect on organism's natural microflora compromising their fitness. Natural microflora contributes to the host immune system, being the "first line of defense" against pathogens, further, they furnish some nutrients that the host could not access alone, for example vitamins, minerals and enzymes (Hansen & Olafsen, 1999; Ozaktas, Taskin, & Gozen, 2012; Yan, van der Gast, & Yu, 2012). The important roles of microflora were already demonstrated by several studies, such as the prevention of infection by pathogenic fungi in crustacean eggs by bacteria producing an antifungal compound (Gil-Turnes & Fenical, 1992) the weight regulation by gut microbiota in mice and Humans (Fraune & Bosch, 2010; Serino, Luche, Chabo, Amar, & Burcelin, 2009) and behavioral patterns monitoring in mammals (Archie & Theis, 2011).

It is known that some environmental stressors such as temperature, oxygen levels, pH or pollutants can have a negative influence weakening these "first line defense" of the organism (Hansen & Olafsen, 1999; Li, Yu, Feng, Yan, & Gong, 2012). However, few is known about the influence of combined effects of UV radiation and chemicals in organism's natural microflora.

Due to the importance of the natural microflora in organism's health and survival, it is important to understand how it is affected by combinations of chemical and environmental factors and what may be the possible consequences. Thus, in order to answer this question, the aim of this work is to study the effects of combined exposure to UV radiation and three xenobiotics on zebrafish (*Danio rerio*) embryos natural bacterial communities.

The zebrafish presents numerous advantages as test organism. Firstly, it has a high reproductive and development rate, having each female hundred eggs in one spawn (Chao et al., 2010). Secondly, both fertilization and development are external, being the eggs and larvae transparent which allows the monitoring of organs, mutant/transgenic lines development and the real-time observation of host-pathogen interaction. Moreover, zebrafish is one of the best described organisms in developmental biology (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995) and have well described and broad molecular tools (Chao et al., 2010; de Esch, Slieker, Wolterbeek, Woutersen, & de Groot, 2012).

The chemicals chosen for this work were triclosan (TCS), potassium dichromate (PD) and prochloraz (PCL).

Triclosan [5-chloro-2-[2,4-dichloro-phenoxy]-phenol; TCS] is a broad spectrum antimicrobial compound present in many contemporary products such as: hand soaps, shower gels, deodorant soaps, screen cream, toothpaste, plastics and some textiles (Dann & Hontela, 2011; Liu, Ying, Yang, & Zhou, 2009; Lubarsky et al., 2012). Although TCS is discarded with domestic wastewater arriving later to treatment stations, these structures are not always able to remove it completely, being this the primary entry route for TCS in the environment. In fact, TCS was already found in aquatic ecosystem and some studies showed their negative effects in aquatic organisms, especially in fish where it can produce loss of equilibrium, quietness, irregular swimming, spinal malformations, blockage of the mouth and abnormal pigmentation (Oliveira, Domingues, 2009; Grisolia, & Soares, 2009; Orvos et al., 2002).

Potassium dichromate ($K_2Cr_2O_7$) was used as a source of chromium (VI) which is an abundant metal widely distributed in terrestrial and aquatic environments (Farag et al.,

2006; Mohan & Pittman Jr, 2006). This compound is used by several industries such as textile, wood preservations and corrosion control, being frequently discarded with other untreated industrial effluents reaching the surface water and representing an environmental threat (Mishra & Mohanty, 2009; Mohan & Pittman Jr, 2006). Metabolic changes, immune system suppression and alterations of growth and health parameters are some of the effects caused by chromium exposure in aquatic organisms (Farag et al., 2006; Prabakaran, Binuramesh, Steinhagen, & Michael, 2006; Roling, Bain, Gardea-Torresdey, Bader, & Baldwin, 2006).

Procloraz (N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]-1H-imidazole-1-carboxamide) is a broad spectrum fungicide that pertains at imidazole family. This compound is widely used in agriculture and horticulture since 1970s being its main application the reduction of plant disease by the inhibition of cytochrome P450, essential in the biosynthesis of ergosterol, a fundamental component in the fungal membrane (Biales, Bencic, Villeneuve, Ankley, & Lattier, 2011). Due to their broad spectrum, this compound can affect also non target organisms, being its effects also observed in fish, where it can produce behavioral disorders and be teratogenic (Domingues et al., 2011; Saglio, Bretaud, Rivot, & Olsén, 2003).

Molecular tools were used in order to characterize the qualitative and quantitative changes in bacterial communities of exposed organisms. Actually, the molecular tolls present numerous advantages such as the high sensitive and specificity of this techniques and fastness in obtaining results, which made them widely used in several areas (Giraffa & Neviani, 2001; Justé, Thomma, & Lievens, 2008).

The objectives of this work were: i) characterize the bacterial communities in zebrafish embryos at 24, 48, 72 and 96 hour post fertilization (hpf) and iii) characterize the combined effects of UV radiation and the chemicals TCS, PD or PCZ in the natural bacterial communities of 48 hpf embryos. The characterization of bacterial communities was done through DNA extraction, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE).

2. Materials and Methods

Test Organisms

The Zebrafish embryos were provided by the facility established at the Biology department from University of Aveiro (Portugal).

Adult fish are maintained in carbon-filtered water at 26 ±1º C, water conductivity of 750 ±50 µs, pH 7.5 ±0.5 and dissolved oxygen at 95% saturation. The fish are feed with commercial artificial diet (ZM 400 Granular) and brine shrimp twice a day and kept in a photoperiod cycle of 16:8h light/dark.

The 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.

Characterization of zebrafish embryos microbial diversity

Zebrafish embryos microflora was characterized in natural conditions (in embryos not subjected to any chemical or UV condition) at 24, 48, 72 and 96 hours post fertilization (hpf). Eggs were collected and placed randomly in petri dishes (90 mm diameter) under standardized culture conditions (100 eggs per petri dish). Every day, at 24, 48, 72 and 96 hpf a sample of embryos was collected and distributed in to five fastprep tubes in an aseptic way (250 µg of sample per tube; approximately 20 eggs). The embryos were then washed with phosphate buffered saline (PBS) according to Karaiskou et al. (2007). A thousand µl of PBS were added to each tube followed by 5 seconds on the vortex and centrifuged at 16000 G, 15 min at 4ºC. The supernatant was discarded and samples were then frozen (-20ºC) until molecular analysis (maximum storage time: 1 week).

Molecular Analysis

The DNA extraction was performed with E.Z.N.A® Soil DNA kit (Omega Biotek) and subsequently amplified by a Nested PCR selecting the sequence of interest, the 16S rDNA.

The primers used were 27F and 1494R (Weisburg, Barns, Pelletier, & Lane, 1991) in the first PCR and 1378R and 984GC (Nubel et al. 1996) in the second. The PCR reagents were obtained from MBI Fermentas and the reaction conducted in the TProfessinal TRIO thermocycler (Biometra).

The amplification program of the first PCR was 5 min at 94ºC in the denaturation step, followed by 25 cycles of DNA denaturation at 94ºC for 45 s, annealing stage at 56ºC for 45 s and an extension step at 72ºC for 1.5 min. The extension phase occurred at 73ºC for 10 min. The second PCR was performed at 94ºC for 4 min in the denaturation step, proceeded by 30 cycles of denaturation at 95ºC for 1min, annealing at 53ºC for 1 min. and extension at 72ºC for 1.5 min. The extension happened at 72ºC for 7 min. An electrophoresis of 1% agarose gel stained with gel red was used to verify the amplification products.

The DGGE gel was prepared with a denaturing gradient ranging from 40 % to 58 % and performed with 1x Tris-acetate-EDTA buffer (TAE) at 60ºC and 70 V for 960 min. The revelation process of the gel was done according to Byun et al. (2009) except that the stop solution used was a Na_2C0_3. A mixture of DGGE-PCR products from different bacterial species was used as DGGE marker.

Zebrafish embryo combined assay

Chemicals

Zebrafish embryos were exposed to concentrations of triclosan (TCS), potassium dichromate (PD) or prochloraz (PCZ) in combination with UV radiation. The concentrations chosen of each chemical were the lowest-observable-effect concentration

(LOEC) and the LOEC/10, corresponding to 0.3 and 0.03 mg/L of TCS; 75 and 7.5 mg/L of PD and 0.6 and 0.06 mg/L of PCZ.

Triclosan (Irgasan, 5-chloro-2-(2,4-dichlorophenoxy)phenol) was acquired from Sigma- Aldrich. The stock solution was prepared dissolving TCS in acetone and completed with ultrapure water. The sub-stock was prepared by diluting the stock in fish system water and test solutions of 0.03 and 0.3 mg/L were set diluting the sub-stock in fish system water.

Potassium dichromate ($K_2Cr_2O_7$) was used as a source of chromium (VI). The solutions of 7.5 and 75 mg/L were prepared diluting a stock obtained by dissolution of potassium dichromate in fish system water.

Prochloraz (1-[N-propyl-N-2-(2,4,6-trichlorophenoxy) ethylcarbamoyl] imidazole I: C15H16Cl3N3O2) PESTANAL ®, (CAS Number: 67747-09-05) was purchase from Sigma-Aldrich Biotechnology LP. A stock solution was obtained dissolving the prochloraz in acetone and completed with fish system water. The test solutions of 0.06 and 0.6 mg/L were prepared dissolving the stock in fish system water.

Ultraviolet radiation

UV levels used in the tests were sublethal levels which do not elicit any mortality in zebrafish embryos according to our previous experiments. The radiation was provided by UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312 nm) and a cellulose acetate filter was used. The filter, previously UV irradiated for 12h, cut off all UV-C radiation and was used in order to minimize the differences in UV radiation intensity passing through the UV lamp.

Intensities of emitted UV radiation were measured with a spectro-radiometer, connected to a monochromator that provided information on energy per nanometer. All the intensities were measured before and after each exposure. Spectral irradiance was obtained by the BenWin+ software (Benthan Instruments, Reading, UK). The different UV intensities were obtained by varying the distances between the petri dishes and the UV lamp.

The UV dose ($J.m^{-2}$) was obtained taking into account the time of exposure and was calculated as: UV dose ($J.m^{-2}$) = UV intensity ($mW.m^{-2}$) × time of exposure (s)/10^3.

Exposure conditions

Three tests were performed: UV radiation combined with TCS, PD or PCL. For each test, nine treatments were used in a full factorial design (using three chemical concentrations and three levels of UV radiation) according to table 8. Newly fertilized eggs were distributed randomly in several petri dishes (60 eggs per petri dish), with the different test solutions (3 replicates were used per treatment). Petri dishes were then placed under the UV lamp in order to receive the desired dose of radiation. Combined exposure to UV light and chemical stress run for 8 hours, then petri dishes was transferred to regular light condition until the end of the test (48 h). Test run under controlled temperature (26 ±1º C) and photoperiod (16:8h light/dark). At 48 h approximately 60 embryos (250 µg of sample) were collected in an aseptic way to three fastprep tubes and washed with PBS according to Karaiskou et al. (2007). A thousand µl of PBS were added to each tube followed by 5 seconds on the vortex and centrifuged at 16000 G, 15 min at 4ºC. The supernatant was discarded and samples were then frozen (-20ºC) until molecular analysis (see section 2- Molecular analysis).

Table 8 - Chemical concentration and UV doses exposure

Combination	Chemical concentration **(mg/L)**	UV dose **($Kj.m^{-2}$)**
UV x TCS	0	0
	0.03	3.10
	0.30	6.53
UV x PD	0	0
	7.5	3.10
	75	12.22
UV x PCZ	0	0
	0.06	3.18
	0.60	6.47

Data analysis

The DGGE was analyzed visually by the observation of alterations in the band pattern (number of bands, relative position in the line and intensity) between treatments. DGGE data was further processed using the Bionumerics software (Aplied Mathematics). Bionumerics output consists of a table containing band position and band intensity of each treatment. These data was then analyzed using Primer 6 software (Clarke & Gorley, 2006). Log(x+1) transformed data was used to calculate the Shannon index. Then, a Bray-Curtis resemblance matrix was done and the matrices visualized by ordination analysis through Non-Metric Multi-dimensional scaling (MDS) of each test.

A Two-way ANOVA test was performed on SigmaStat software (Spss, 2004) to assess effects of UV radiation and chemicals. Analyses were performed with a significance level of 0.05.

3. Results

Characterization of zebrafish embryos microbial diversity

In order to assess differences in zebrafish embryos micoflora along their early development, a DGGE band pattern was obtained for 24, 48, 72 and 96 hours post fertilization embryos. The external lanes (Fig. 13, M) represent a marker, while the middle lanes represent the samples (3 replicates per sample). Each line contains different bands representing different bacterial communities which were separated in the gel based on their different denaturing DNA characteristics. Different band intensities mean different quantity of bacterial community, (intense bands have a higher amount of bacterial communities and the soft bands have a lower amount of bacteria).

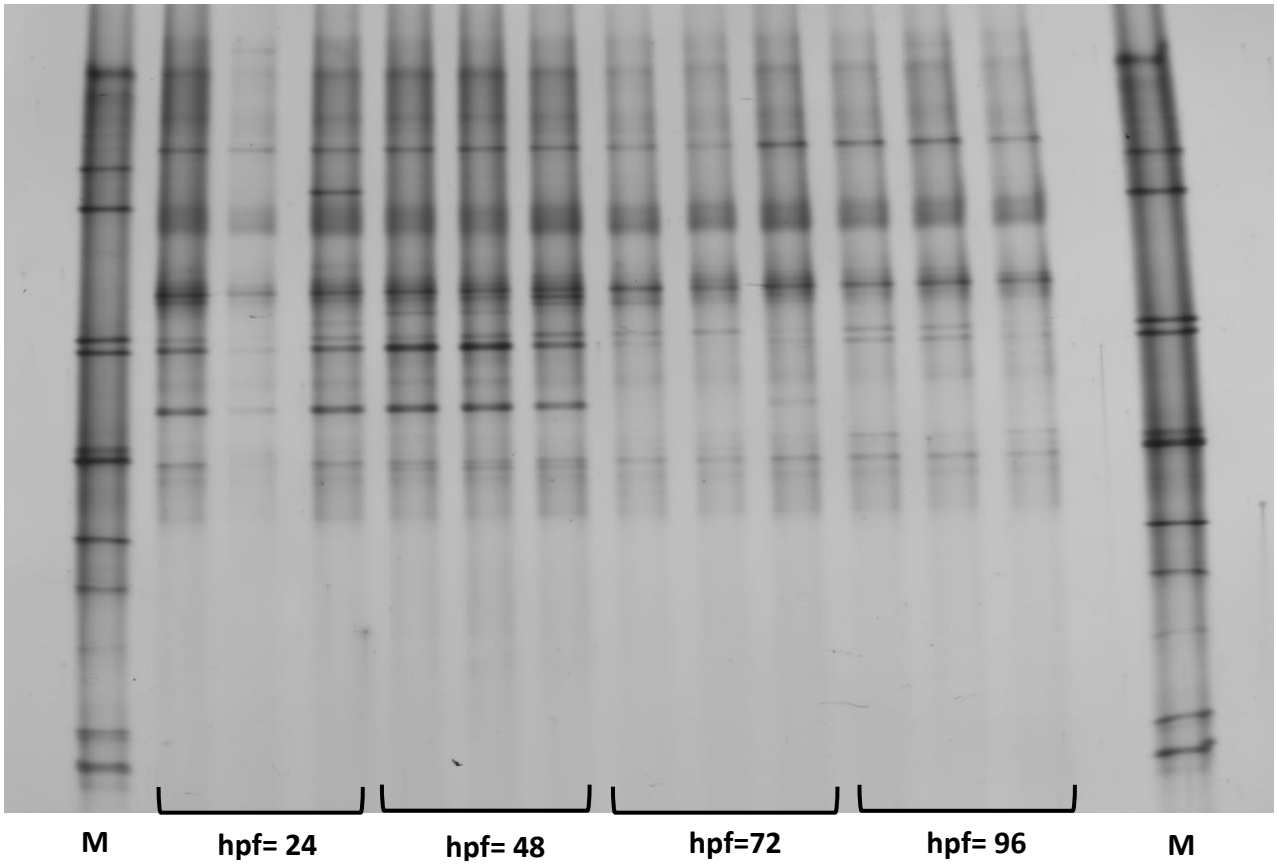

Figure 13 - DGGE image representing zebrafish embryos bacterial communities at 24, 48, 72 and 96 hours of development. M refers to marker.

The gel obtained (Fig. 13), shows that the number of bands is much higher at 24 and 48 hpf than at 72 and 96 hpf. Furthermore, the intensity of bands at 48 hpf is higher than at 72 and 96 hpf. The MDS (Fig. 14) confirms the visual analysis of the gel showing two distinct groups formed spatially; one group corresponding to the data of 24 and 48 hpf and the other corresponding to the data of 72 and 96 hpf.

The Shannon index (Table 9) confirms the same trend and was higher to 48 hpf embryos (2.35) indicating higher diversity of bacterial communities at this stage of development. Thus, in the following combined tests, microbial analyses were performed at 48 hpf.

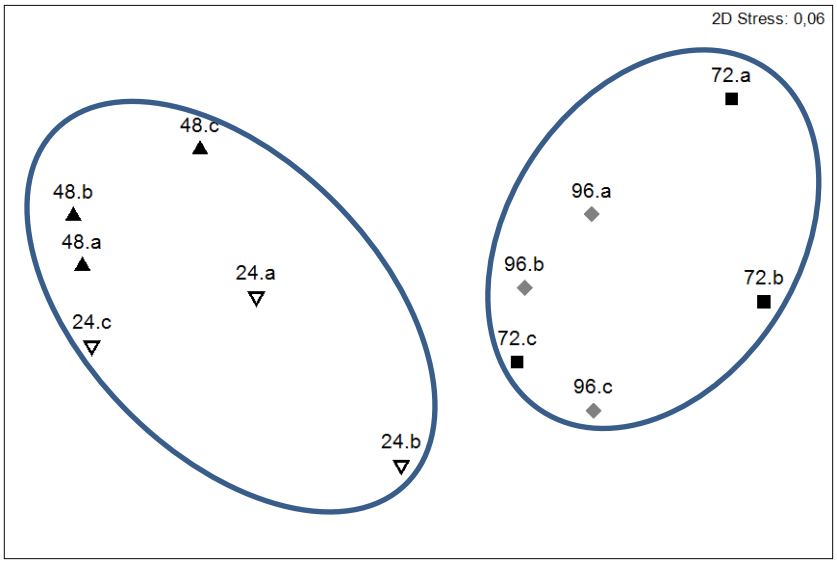

Figure 14 - Ordination diagram (MDS) of bacterial community of zebrafish embryos at 24 (▽), 48 (▲), 72 (■) and 96 (◆) hours post fertilization.

Table 9 - Shannon index calculated for bacterial communities of zebrafish embryos of different ages. (S =total number of taxa)

hpf	S	Shannon index
24	13	2.19
48	14	2.35
72	9	1.84
96	9	1.89

Characterization of zebrafish embryos bacterial communities in combined exposure

UV effect

In the DGGE images a clear effect of UV radiation alone in the bacterial communities can be observed (Fig. 15, 18 and 21; treatments in which chemical concentration is zero). DGGE corresponding to embryos subjected to UV radiation show a substitution of bands (some disappeared and others show up) and an increase in the intensity of some bands when compared to control (normal light). Moreover, differences in the composition of bands were also observed between the lowest and the highest UV intensity treatments (differences in the position of the bands between them can be observed). These results can also be observed in the MDS analysis where the groups corresponding to different UV intensities appear spatially differentiated (Fig. 16; ○) Shannon index, however, was not significantly different between UV treatments, meaning that no overall differences in bacterial diversity were observed (Fig 17, 20 and 23).

TCS and UV effect

In the DGGE resulting from combined UV radiation and TCS exposure, and for those organisms exposed to highest dose of UV (6.53 $Kj.m^{-2}$) a new band (↗) shows up corresponding to UV effect that is repeated in all combinations with the same UV dose. In organisms exposed to lower TCS concentration (0.03 mg/L) occurs the increase in the intensity of bands (*↗) already observed in the control treatment (0 mg/L), while in those organisms exposed to higher concentration (0.3 mg/L) of TCS a new band appears (**↗). In the treatments where highest UV doses and TCS concentrations are combined, response pattern of both stressors can be identified (1↗ and 2↗).

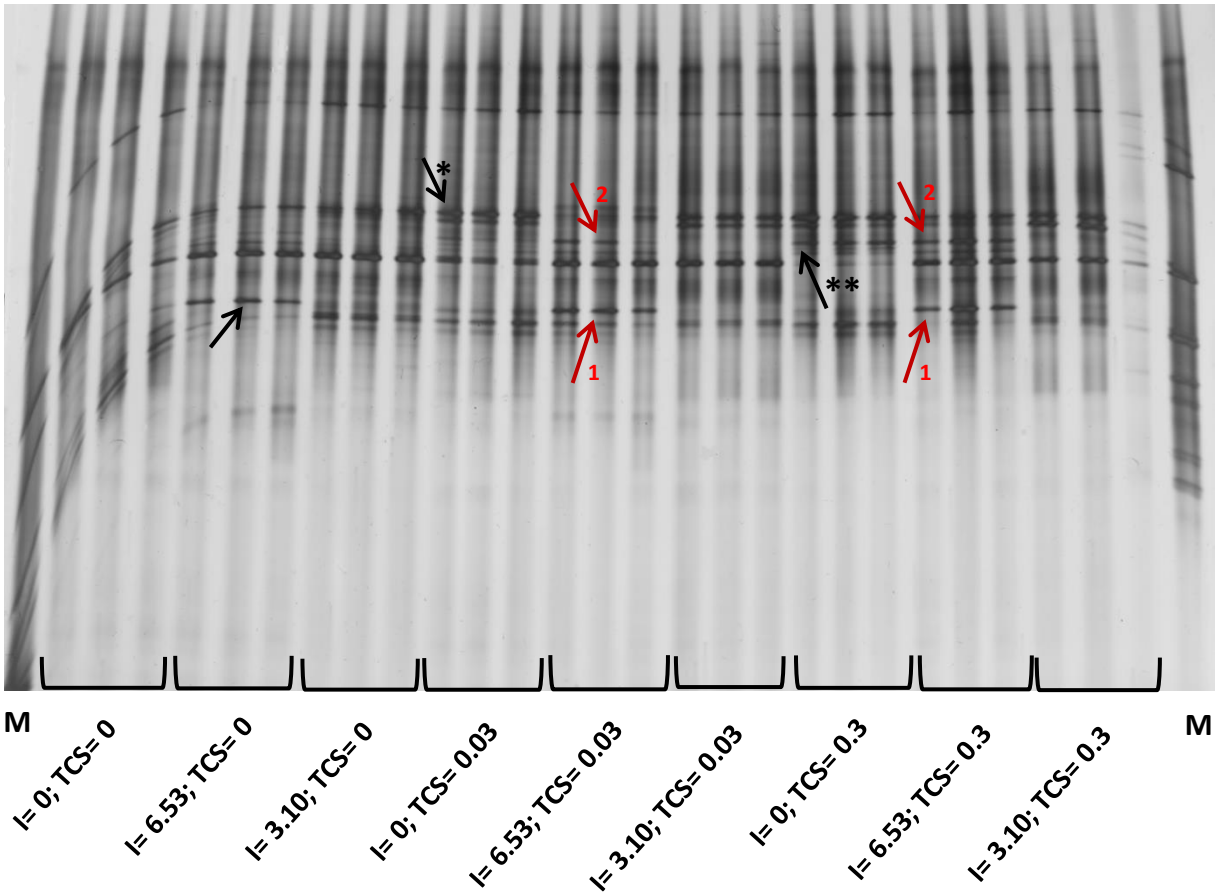

Figure 15 - DGGE image of combined effect of UV radiation and triclosan. Units are in mg/L to TCS concentration and Kj.m^{-2} to UV dose. M refers to marker.

The ordinary diagram of bacterial community seems does not show a clear spatial distribution of treatments probably because 2 of the replicates of the control (0 mg/L of TCS and 0 of UV intensity) seem to be very different (distant) from all other samples, decreasing the capacity of differentiation between treatments (Fig. 16). Moreover, analyzing changes on bacterial communities' diversity (Shannon index, Fig. 17), no effects in bacterial diversity could be observed due to UV (p=0.408) or TSC (p=0.228). However, the 2-way ANOVA indicated an interaction between these two factors (p=0.004) (Table 10).

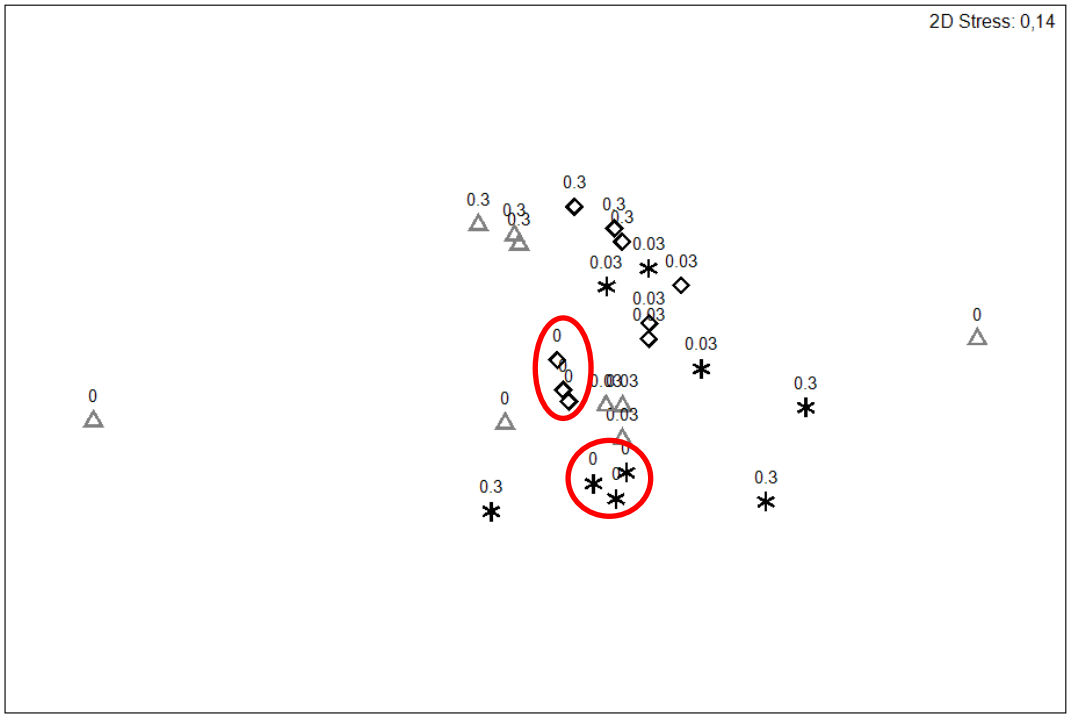

Figure 16 - Ordination diagram (MDS) of bacterial community by treatment in combined test of UV radiation with TCS. Triangles correspond to 0 Kj.m^{-2} UV dose; asterisks to 3.10 Kj.m^{-2} and diamonds to 6.53 Kj.m^{-2}. Numbers above symbols refer to TCS concentration in mg/L.

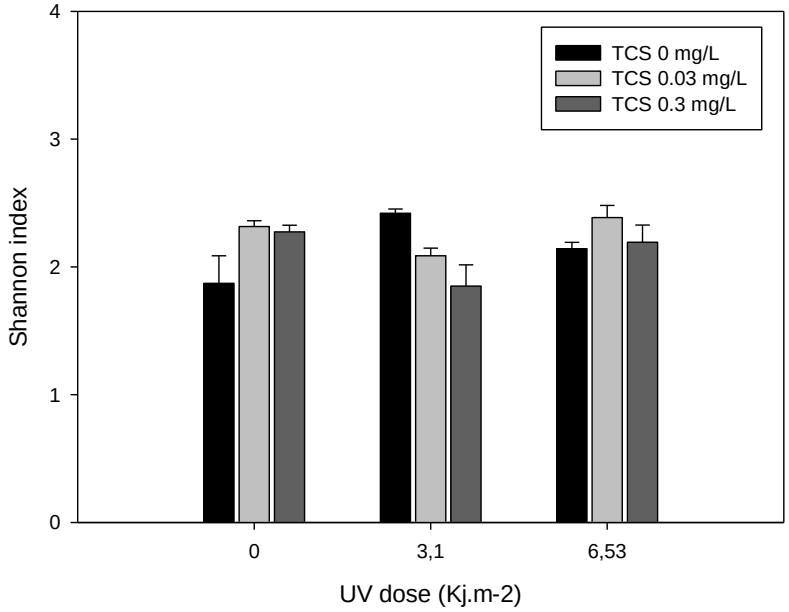

Figure 17 - Shannon Index in combined exposure to UV radiation with TCS

PD and UV effect

The visual analysis of the gel (Fig. 18) indicates a clear effect of the metal chromium with a drastic reduction in the number of bands at PD concentrations of 75 mg/L (reduction of bacterial diversity). At concentration of 7.5 mg/L new bands (↗) can be observed while others disappeared when compared to 0 mg/L treatment. In combined treatments of the lowest UV dose (3.10 $Kj.m^{-2}$) with 7.5 mg/L or 0 mg/L PD it is clear the increase of intensity of several bands while others completely disappear. The data corresponding to treatments without PD (UV exposure only) reveal a constant band pattern, however in treatment of lowest UV dose (3.10 $Kj.m^{-2}$) there is a decrease in the number of bands which represents a loss of diversity of bacterial communities.

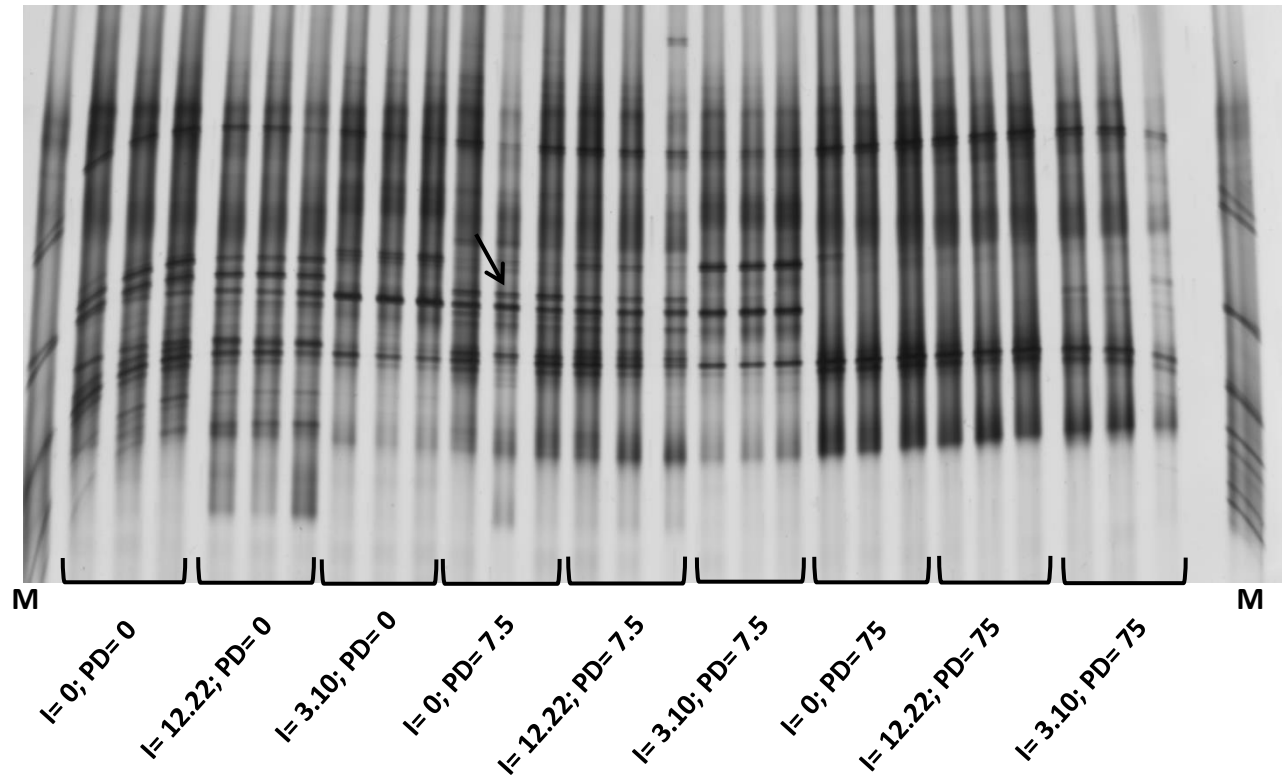

Figure 18 - DGGE gel image of combined effect of UV radiation and potassium dichromate. Units are in mg/L to PD concentration and $Kj.m^{-2}$ to UV dose. M refers to marker.

MDS also revealed the marked effect of PD with a clear separation of two groups, one composed by all treatments including 75 mg/L of PD (left side of image) and the other group composed by treatments 0 and 7.5 mg/L of PD (right side of image), independently of the level of UV radiation. Inside of each group smaller sets can be identified. Treatments of 75mg/L can be separated by the UV dose, (zero and higher intensity at the top of the image ( ), and lower UV dose are at the bottom of image ( ). Treatments of 0 mg/L PD are located in the middle of image ( ) and treatments of 7.5 mg/L of PD are distributed around this group.

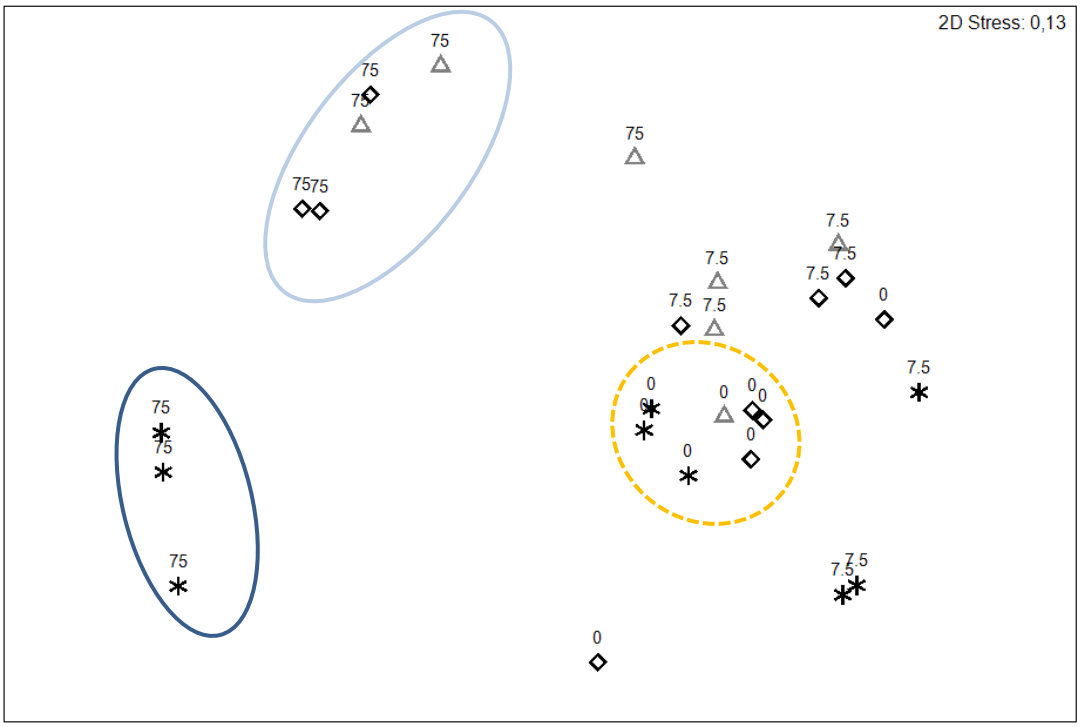

Figure 19 - Ordination diagram (MDS) of bacterial community by treatment. Triangle correspond to 0 Kj.m^{-2} UV dose; asterisks to 3.10 Kj.m^{-2} and diamonds to 12.22 Kj.m^{-2}. Numbers above symbols refer to PD concentration in mg/L.

The change of bacterial communities by PD is also accompanied by the change of bacterial diversity ($p<0.001$) (Fig. 20). Interestingly, at 7.5 mg/L of PD the Shannon index was lower under low UV doses than with the highest UV dose (which agrees with the MDS). Combination of UV dose and PD also indicated a significant interaction ($p<0.001$) with the UV effect depending on what level of PD is present (Table 10).

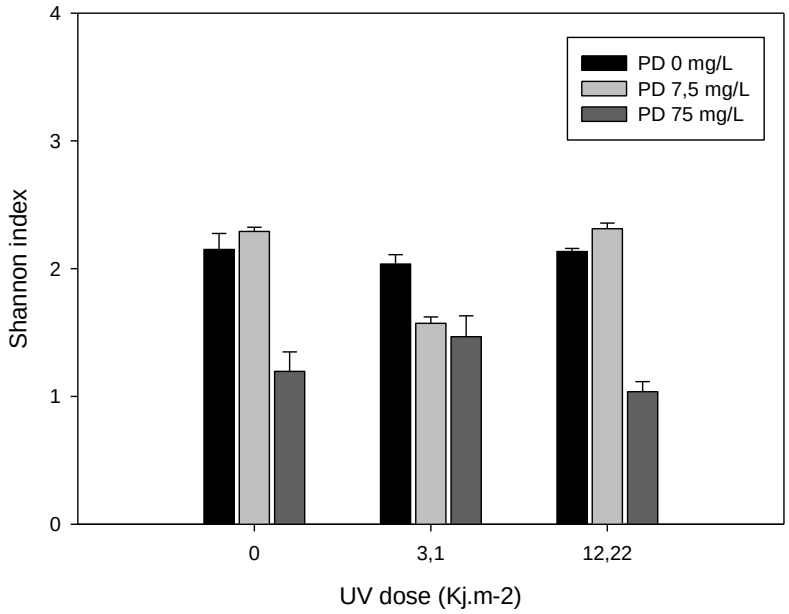

Figure 20 - Shannon Index in combined exposure to UV radiation with PD

PCZ and UV effect

Relative to DGGE image, PCZ seems to have an effect only at the highest concentration where an increase in the intensity of some bands (↗) and the appearance of others (↗) can be observed. In the combinations with UV radiation and both concentration of PCZ the pattern observed for both stressors can be identified.

Spatial distribution (Fig. 22) shows two different groups; one constituted by all the treatment without UV radiation (○) and the other with the treatments of UV radiation (○). Thus, indicating a distinct effect of UV radiation. However within each group, a gradient of PCZ can also be identified (higher doses at the bottom, lower doses and no chemical at the top).

Analyzing the Shannon index (Fig. 23), the diversity of bacterial community seems do not have differences in control group (0 mg/L of PCZ) and neither in treatment of lowest concentration of PCZ (0.06 mg/L), while in treatments of 0.6 mg/L of PCZ the bacterial diversity seems to decrease with the UV dose. The Two-way ANOVA indicated

no effect of UVs in bacterial community diversity (p=0.135) whereas PCZ showed to have a significant effect (p<0.001).

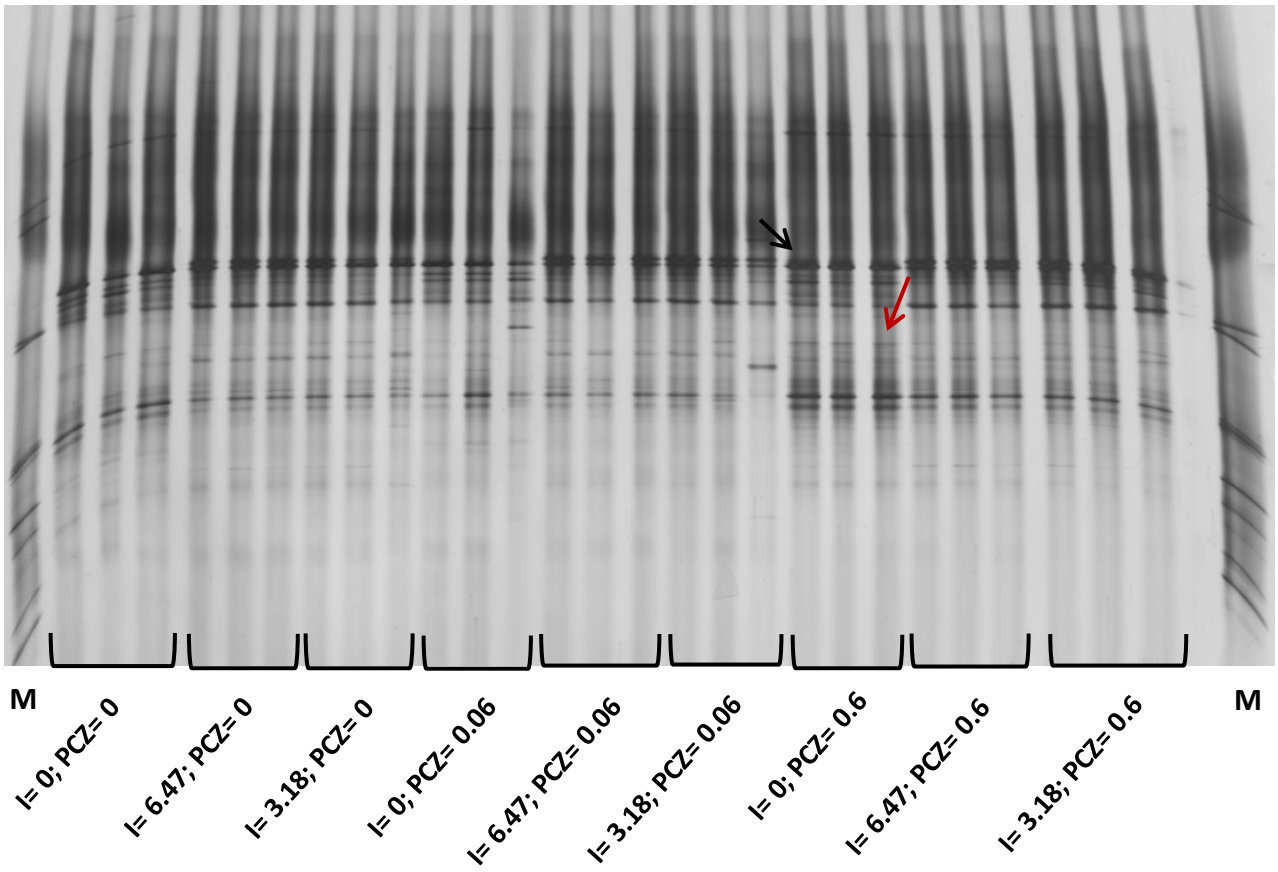

Figure 21 - DGGE gel image of combined effect of UV radiation and prochloraz. Units are in mg/L to PCZ concentration and Kj.m^{-2} to UV dose. M refers to marker.

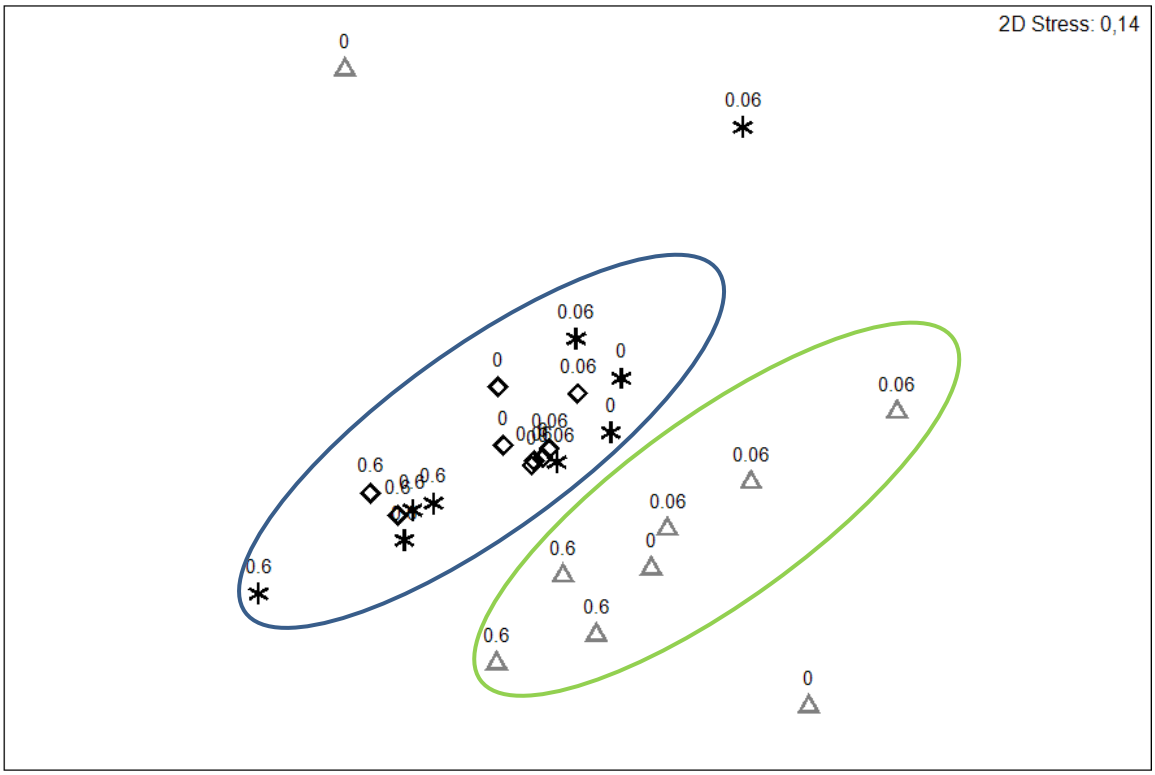

Figure 22 - Ordination diagram (MDS) of bacterial community by treatment. Triangles correspond to 0 Kj.m^{-2} UV dose; asterisks to 3.18 Kj.m^{-2} and diamonds to 6.47 Kj.m^{-2}. Numbers above symbols refer to PCZ concentration in mg/L

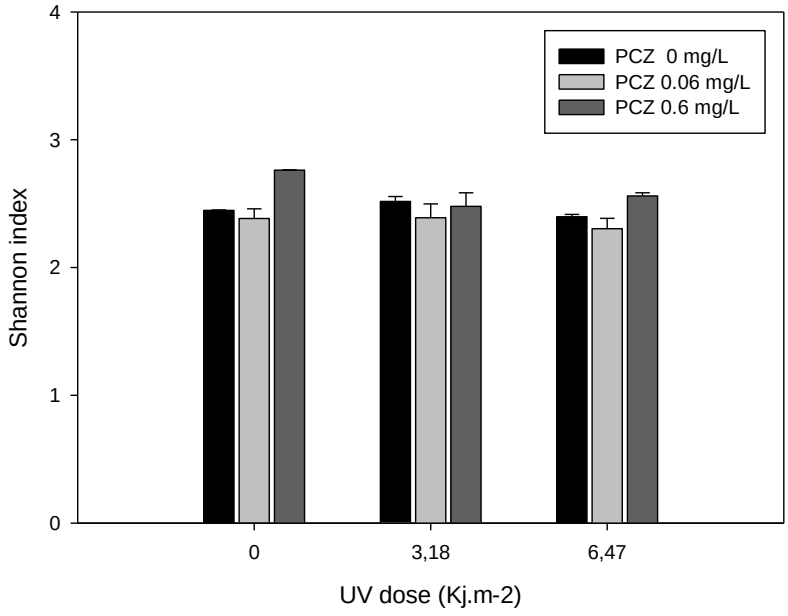

Figure 23 - Shannon Index in combined exposure to UV radiation with PCZ

Table 10 - Two-Way ANOVA analysis of microbial diversity (Shannon Index) in combined test of UV radiation and TCS, PD or PCZ

	TCS			PD			PCZ		
	Degrees of Freedom	**F**	**P**	**Degrees of Freedom**	**F**	**P**	**Degrees of Freedom**	**F**	**P**
UV radiation	2	0.941	0.408	2	3.011	0.074	2	2.239	0.135
Chemical	2	1.609	0.228	2	77.811	<0.001	2	10.488	<0.001
UV radiation X Chemical	4	5.552	0.004	4	10.797	<0.001	4	2.115	0.121

4. Discussion

Characterization of zebrafish embryos microflora

Zebrafish embryos showed to have different microflora diversity during his lifespan. During the egg stage, at 24 and 48 hpf, the embryos showed to have a higher microbial diversity than during larvae stages, at 72 and 96 hpf. Actually, the eggshell is constituted by glycoproteins which are suitable for the adhesion of bacteria. Bacteria of the surrounding environment adhere to the eggshell and constitute a barrier against possible pathogens (Hansen & Olafsen, 1999). Once the embryo hatch, the adherent bacteria stay in the eggshell which explains the loss of microbial diversity in the passage from the egg to larvae stage.

Characterization of zebrafish embryos microflora under UV and chemical exposure

Through the analysis of DGGE images, the effect of UV radiation is clear, with the enhancing of intensity of some bands and the appearance of others. This effect may be due to the growth of UV radiation tolerant bacteria (Joux, Jeffrey, Lebaron, & Mitchell, 1999). Some authors already proved that bacteria have different sensibility to UV radiation (Arrieta, Weinbauer, & Herndl, 2000; A. L. Santos et al., 2012). This difference in sensibility may lead to the weakening of several susceptible communities while more resistant communities become predominant, suggesting that UV radiation may act as a selector (A. Santos et al., 2011). This selection will change the community structure and functioning which may have an effect in the host organism.

In all the tests, however, UV radiation did not have a significant effect on bacterial community diversity, expressed by the Shannon index. Indeed, the Shannon index only gives information about the community's diversity, leaving aside their composition/structure. Although we did not detect differences in bacterial communities' diversity, the communities' structure was affected.

TCS alone seems to produce an effect in bacterial communities. At lowest concentration an increase of intensity of some bands which are also present in the control can be seen; moreover, at the highest concentration new bacterial communities show up. TCS is a broad spectrum antimicrobial agent having as target the cytoplasmic membrane of bacteria. Given that in our work concentrations below the minimum inhibitory levels where used (Panagakos, Volpe, Petrone, DeVizio, & Davies, 2005), the natural bacteria communities may have been only weakened. The weakening of these bacteria species allowed the arising of other and the consequent change in band pattern.

Although a shift in some bacterial species was evident by gel analysis, no differences in the Shannon index were observed. Thus, the Shannon index revealed do not be a good effect indicator, as it only takes into account the overall diversity and not shifts in the communities' structure.

PD showed to have a strong effect in zebrafish embryos bacterial community. Embryos showed a dose dependent reduction of bacterial communities. Cr (VI) is highly

toxic, producing mutagenic effects. It easily crosses cellular membrane and increases free radicals which will cause DNA changes (Dhal, Thatoi, Das, & Pandey, 2013). This mode of action explains the significant loss of microbial communities in embryos exposed to the highest concentration of PD (75 mg/L) as represented by the lower Shannon Index. Indeed, the loss of bacterial diversity under exposure to chromium was also observed in soil communities (Viti & Giovannetti, 2001).

Moreover, in combined treatments with the lowest doses of both stressors, occurred the increase of some bands intensity which did not occur in the other UV doses suggesting an interactive effect. Furthermore, an interactive effect seems also to occur in combined treatments of lowest UV dose and highest PD concentration with the resistance of some communities. These results may suggest an antagonistic effect when UV radiation is present in lower doses. The Shannon index also revealed significant differences in bacteria diversity to combined exposure.

PCZ seems do not affect the bacterial communities at the lowest concentrations tested, showing a band pattern similar to the control, but, at the highest concentration it seems to occurs an increase in band intensity. PCZ is a broad spectrum fungicide with fungi as organism target, nevertheless some authors already showed its effect on non-target organisms such as bacteria (Munoz-Leoz et al., 2013; Yang, Hamel, Gan, & Vujanovic, 2012).

In the combined exposure, the band pattern is caused mainly by UV doses effect, however, two way ANOVA revealed that there is only effects on PCZ exposure.

Differences between the gel image analyses and Shannon index turn difficult data interpretation. The shift of some bacterial communities will not produce an effect on the diversity index even though it is an important effect.

Nevertheless, in our work, effects in the bacterial communities were observed even in very low concentrations (LOEC/10) of each chemical which did not produce any effect in the host organism in conventional ecotoxicology testes. These are important results as effects in microbial communities are very rarely contemplated in studies of risk assessment. Disturbances in the equilibrium of natural bacterial communities of embryos

may compromise the fitness and health of populations with unknown consequences at community level.

Future works should be made in order to clarify the interactive effects of UV radiation with the chemicals tested. Shannon index showed do not be a good effect indicator revealing the need to find another ways to express objectively the changes in communities. Other molecular analyses such as pyrosequencing may be an alternative to deeper understand DGGE results because it would reveal the constitution of communities identified in the gel.

Furthermore in our work high UV doses and short exposure times were used in the experimental design. In future works, more realistic exposure scenarios should be used including lower UV doses and longer exposure periods. Moreover, it would also be interesting to understand if the changes observed in embryos are reversible or not and what their long term consequences (what effects will be observed in adults?).

5. Conclusion

Along their development, zebrafish embryos present different bacteria communities and diversity. In the egg stage the microbial diversity is higher than in the larvae stage which is explained by the adherent bacteria to the eggshell.

Further, the natural bacteria community of organisms can be affected by several factors such as UV radiation and chemicals. In our work, effects of UV radiation, TCS, PD and PCZ were observed, however interactive effects of both types of stressors were difficult to assess.

In all tests, was revealed an effect on communities' structure performed by UV radiation with the emerging of new communities. In combined test of UV radiation with TCS the main pattern effect was the shift of bacterial communities, while in the combined test of UV radiation with PD there was a big loss of bacterial communities at higher PD

concentration. PCZ seems do not have a clear effect being the main band pattern formed by UV radiation action.

Although sometimes Shannon index does not showed significant differences between treatments, through the gel image and MDS analysis we could see changes in bacterial communities structure what difficult the interpretation of the data. Thus, there is need more studies to conclude about possible interactions.

Changes in natural bacterial communities may produce changes in activity, dynamics and function of these communities. Thus, natural communities are essential to the eggs health and survival. Furthermore, changes in bacterial communities' structure may lead to the degradation of the egg shell or even the egg did not hatch compromising their development.

Aknowledgments

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

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Chapter 4

CONCLUSION

In natural environment, organisms are subjected to multiple stressors which can affect organism's health and survival. Further, these stressors can interact with each other producing synergistic or antagonistic effect.

In this work was demonstrated the interactive effect of UV radiation and TCS, PD and PCZ on zebrafish survival and natural bacteria communities.

The results showed that different interactive responses can occur. In combined test of UV radiation with TCS was observed a synergistic effect when UV radiation is the dominant stressor. Indeed, this is the worst scenario once it is the most likely to occur in nature. The combined test of UV radiation with PD revealed that antagonism may occur at low stress levels while synergism is expected at high doses. Combined test o UV radiation with PCZ revealed antagonism when UV radiation is the dominant stressor.

The natural bacteria communities of zebrafish also showed to be affected by UV and chemicals even at low concentration (LOEC/10). The main pattern effect of UV radiation was the change of bacterial communities' structure with the arising of new communities. TCS and PD also revealed to affect bacteria with the shift and the loss of communities by each chemical respectively. PCZ seems do not affect bacteria communities. Due to the difficult in data interpretation nothing can be concluded about possible interactions.

Our results highlight to the importance of including environmental factor, such UV radiation as additional stressors in future works. Interactive effects studies may be into account not only organisms' survival but also changes in bacterial communities once changes in natural bacterial communities may compromise eggs health and survival. Additional works may be done in order to predict long term effects on organisms at fitness, survival and reproductively levels.