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Study of the degradation of UV-filters in chlorinated waters and toxicological assessment.

Dissertation of candidature for the master degree in Environmental Toxicology and Contamination submitted to Biomedical Science Institute Abel Salazar of University of Porto

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“Se sábio não julgas ser
ainda bem que te conheces.
Se julgas tudo saber
o teu saber empobreces.”

- Alberto Sá (1913-2004)

Acknowledgment

To Professor Joaquim Carlos Gomes Esteves da Silva and to Margarida da Silva Miranda for all the guidance and support along this project.

To Professor Jorge Miguel de Ascenção Oliveira, Professor Isabel Filipa Martins de Almeida and to Professor José Paulo Cabral de Sousa e Silva from Faculty of Pharmacy of the University of Porto for all the support about the toxicological assays with zebrafish. I also really thank Ana Rocha for all the time spent with me and all the transmitted knowledge.

To Dr. Zélia Azevedo for all the help with the LC-DAD-MS analysis.

To all my colleagues from the laboratory for all the help and the friendship. A special thanks to Diana Crista for all the support along all the work.

To my father for giving me the opportunity to continue with my studies and for encouraging me every day to learn more and more. To all my family: without you, I could never get this far.

To Manuela Barros for always being by my side in any situation: your support was crucial and I am eternally grateful.

To all my friends: you make life so much more special. To Pedro Fernandes for showing me what was invisible to my eyes: you are a really special person!

Thank you!

Abstract

Pharmaceuticals and Personal Care Products (PPCPs) is a category of compounds where are included pharmaceutical drugs, cosmetic ingredients, food supplements, shampoos, lotions and sunscreens cosmetics. These products contain a number of organic compounds such as UV-filters, fragrances, antimicrobials, preservatives and insect repellants also collectively referred as PPCPs. PPCPs have been found in surface water such as lakes, rivers and sea, waste water and tap water and also in bathing water and swimming pool water. Disinfection of swimming pool water is essential to inactivate pathogenic microorganisms. However chlorine based disinfectants, the most commonly used, react with the organic matter present in water leading to the formation of disinfection byproducts (DBPs) which are known to be associated to adverse health effects. PPCPs may also suffer transformation/degradation caused by sunlight UV radiation. The present study aims improve the knowledge about the transformations of UV-filters occurred in swimming pools. The present work had two major objectives: (i) the assess of the stability of four UV-filters (benzyl salicylate, phenyl salicylate, 4-methyl benzilydene camphor and octocrylene) in chlorinated water, (a) evaluating their stability in water samples with different pH values and different free chlorine conditions; (b) to determine their half-lives; (c) tentatively identify the major DBPs and (d) to study their stability under time, and (ii) assess the toxicity of another UV-filter (4-methoxy-4‘-tert-butyldibenzoylmethane) comparing the toxicity caused by the parental compound to the toxicity of its DBPs acutely exposing larvae of zebrafish (*Danio rerio*) to several concentrations of 4-methoxy-4‘-tert-butyldibenzoylmethane and corresponding DBPs solution. The effect of these compounds regarding mortality and anatomic abnormalities was assessed. The reactions between the UV-filters and chlorine were followed by HPLC-MS. Benzyl salicylate and phenyl salicylate reacted with chlorine yielding three DBPs each one. 4-methyl benzilydene camphor and octocrylene were found to be stable under the experimental conditions used. It was not possible to conclude if 4-methoxy-4‘-tert-butyldibenzoylmethane and its DBPs at 1 mg/L have impact on the larvae although it was observed some abnormalities at 0.1 mg/L of DBPs.

Keywords: UV-filters, personal care products, chlorination, chlorinated byproducts, benzyl salicylate, phenyl salicylate, 4-methyl-benzylidene camphor, 4-methoxy-4‘-tert-butyldibenzoylmethane, zebrafish

Resumo

Produtos farmacêuticos e outros produtos de cuidado corporal (PPCPs, do Inglês *Pharmaceuticals and Personal Care Products*) são uma categoria de produtos que inclui fármacos, ingredientes de cosméticos, suplementos alimentares, champôs, loções, protectores solares entre outros. Estes produtos contêm vários compostos orgânicos como filtros UV, fragrâncias, compostos com propriedades antimicrobianas, conservantes e repelentes de insecros que se incluem também na categoria de PPCPs. Vários PPCPs têm sido encontrados em águas de superfície como lagos, rios e mar, águas de esgoto, água da torneira, bem como também em águas de piscina. A desinfecção da água das piscinas é fundamental para inactivar os microrganismos patogénicos. No entanto, os desinfectantes à base de cloro, que são os mais usados, reagem com a matéria orgânica presente na água levando à formação de produtos de desinfecção (DBPs, do Inglês *Disinfection Byproducts*) que podem estar associados a efeitos nefastos para a saúde. PPCPs também podem sofrer transformações resultantes da exposição à radiação UV solar. Este estudo visa aumentar o conhecimento sobre os processos de transformação sofridos pelos filtros UV em piscinas. Os seus dois maiores objectivos passam por (i) avaliar a estabilidade de quatro filtros UV (benzil salicilato, fenil salicilato, 4-metilbenzilideno cânfora e octocrileno) em água clorada (a) avaliando a sua estabilidade sob diferentes valores de pH e diferentes concentrações de cloro, (b) determinando os tempos de semi-vida, (c) identificando os principais DBPs (d) estudando a sua estabilidade ao longo do tempo, e (ii) avaliar a toxicidade de um outro filtro UV (4-metoxi-4‘-tert-butildibenzoilmetano) comparando a toxicidade causada pelo composto parental com a toxicidade causada pelos seus DBPs, expondo de forma aguda larvas de peixe-zebra (*Danio rerio*) a várias concentrações de 4-metoxi-4‘-tert-butildibenzoilmetano e correspondentes DBPs. Os efeitos destes compostos foram avaliados ao nível da mortalidade e malformações. As reacções entre os filtros UV e o cloro foram seguidas por HPLC-MS. Benzil salicilato e fenil salicilato reagiram com o cloro formando três DBPs cada um. 4-metilbenzilideno cânfora e octocrileno mantiveram-se estáveis sob as condições experimentais usadas. Não foi possível concluir se 4-metoxi-4’-tert-butildibenzoilmetano e os seus DBPs tiveram impacto nas larvas à concentração de 1 mg/L apesar de se ter observado alguma mal formações a concentrações de 0.1 mg/L dos DBPs.

Index

Index of figures

Chapter II: State of art

Index of tables

List of abbreviations

4-MBC: 4-Methyl Benzilydene Camphor

dpf: Days post Fertilization
hpf: Hours post Fertilization
lw: Lipid Weight
ppm: Parts per Million
vs: Versus

ANT: Antipyrine
BCF: Bioconcentration Factor
BDM: 4-Methoxy-4'-Tert-Butyldibenzoylmethane
BP-3, **BP3**: Benzophenone-3
BP-4, **BP4**: Benzophenone-4
BP-8, **BP8**: Benzophenone-8
BzS: Benzyl Salicylate
C: Concentration
C_0**:** Initial Concentration
Ctrl: Control
DAD: Diode-Array Detection
DBPs: Disinfection Byproducts
DOM: Dissolved Organic Matter
EHDPABA: 2-Ethylhexyl-p-Dimethylaminobenzoate
EHMC: Ethylexyl Methoxycinnamete
ES: 2-Ethylhexyl Salicylate
GC: Gas Chromatography
HAA: Haloacetic Acids
HBQs: Halobenzoquinones
HPLC: High-Performance Liquid Chromatography
HPLC-MS: HPLC with Mass Spectrometry
HPLC-UV-DAD: High-Performance Liquid Chromatography with UV-Diode-Array
IAMC: Isoamyl Methoxicinnamte
LC: Liquid Chromatography
MS: Mass Spectrometry
NOECs: No Observable Effect Concentrations

N-DBPs: Nitrogenous-DBPs

OC, **OCT:** Octocrylene

OS: Octyl Salicylate

PABA: *p*-Aminobenzoic Acid

PBSA: Phenyl Benzimidazole Sulfonic Acid

PCPs: Personal Care Products ()

PPCPs: Pharmaceuticals and Personal Care Products

PS: Phenyl Salicylate

R: Correlation Coefficients

ROS: Rreactive Oxygen Species

UV: UltraViolet

UV/Vis: UltraViolet-Visible Detection

List of oral communications presented at scientific congresses

"Degradation of UV-filters benzyl salicylate and phenyl salicylate in chlorinated waters.", IJUP'14 – 7th meeting Investigação Jovem da Universidade do Porto, February 20014

"Study of the Photostability of UV-filters 4-Methylbenzylidene Camphor and Octocrylene in Chlorinated Water", 4PYChem – 4th Portuguese Young Chemists Meeting, April 2014

Chapter I

Introduction

1. Introduction

1.1. Motivation

Pharmaceuticals and Personal Care Products (PPCPs) is a category of compounds where are included pharmaceutical drugs, cosmetic ingredients, food supplements and other products like shampoos, lotions and sunscreens cosmetics (Shen and Andrews, 2011). Sunscreens cosmetics are any cosmetic which contains UV-filters in its formulation to protect human skin from the solar UV radiation since they absorb, reflect and/or scatter UV radiation with a wavelength between 320 and 400 nm for UVA and between 290 and 320 nm for UVB (Negreira *et al.*, 2008; Salvador and Chisvert, 2005; Santos *et al.*, 2012).

PPCPs have been found in surface water such as lakes, rivers and sea, waste water and tap water (Giokas *et al.*, 2004; Poiger *et al.*, 2004; Sui *et al.*, 2011; Westerhoff *et al*., 2005). In the case of pharmaceuticals the main reason for this is that during the wastewater treatment, the parental compounds are not totally removed and, in several cases, they also suffer biodegradation and biotransformation (Onesios and Bouwer, 2009). Then, the release of the effluents in the environment leads to the occurrence of PPCPs and derivatives in the locations above mentioned. PPCPs have been also found in bathing waters and swimming pool water due their use by swimmers (Lakind, 2010) by washing bath effect during bathing and swimming activities (Santos *et al.*, 2012). The problem is that, as in drinking water, the chlorine used in the disinfection process reacts with these compounds generating chlorinated byproducts (DBPs) that may possess enhanced toxicity (Buth *et al.*, 2007; Lakind, 2010, Richardson *et al.*, 2010).

Since UV-filters are designed to absorb a large amount of solar energy, it is probable that their fate in natural waters is controlled by solar mediated mechanisms. However, the information in the literature about photochemistry of UV-filters in illuminated aqueous solutions and in natural waters is still limited.

Some of them have estrogenic activity (Morohoshi *et al.*, 2005; Kunz and Fent, 2006) and are phytotoxic (Rodil *et al.*, 2009) with their toxicity altered by solar irradiation (Rodil *et al.*, 2009, Hayashi *et al.*, 2006). Some UV-filters can photogenerate reactive oxygen species (ROS) (Allen *et al.*, 1996a; Allen *et al.*, 1996b; Inbaraj *et al.*, 2002) that may damage biomacromolecules. For example, phenyl benzimidazole sulfonic acid (PBSA), a widely used UV-filter, can photogenerate $^1O_2$ and $O_2^{-\cdot}$ (Inbaraj *et al.*, 2002) causing DNA damage (Stevenson and Davies, 1999). So, it is important to understand the

photochemical behavior and fate of sunscreens to assess their ecological risk (Zhang *et al*., 2010).

1.2. Goals

The present work had two major objectives: (i) the assess the stability of four UV-filters commonly used in personal care products (PCPs): benzyl salicylate (BzS), phenyl salicylate (PS), 4-methyl benzilydene camphor (4-MBC) and octocrylene (OC) in chlorinated water, in conditions similar to those existent in swimming pools, (a) evaluating their stability in water samples with different pH values and different free chlorine conditions; (b) to determine their half-lives; (c) tentatively identify the major DBPs and (d) to study their stability under time, and (ii) assess the toxicity of an UV-filter whose chlorination reaction was already studied by Santos *et al*., (Santos *et al*., 2013) (4-methoxy-4'-tert-butyldibenzoylmethane - BDM) comparing the toxicity caused by the parental compound to the toxicity of its DBPs acutely exposing larvae of zebrafish (*Danio rerio*) to several concentrations of BDM and corresponding DBPs solution resulting from chlorination reaction. The effect of these compounds regarding mortality and anatomic abnormalities was assessed. This toxic assay was performed on an experimental basis giving a first approach on the study of BDM´s toxicity.

1.3. Structure of the thesis

The present thesis is subdivided in six chapters:

- Chapter I: where the motivation, main goals and the structure of the thesis are introduced.
- Chapter II: State of the Art, where is presented a review of the literature about the presence of PPCPs in chlorinated waters, the reaction between them and chlorine and the environmental and health impacts of PPCPs and their DBPs. In this part, is present a book chapter (Occurrence of Personal Care Products and Transformations Process in Chlorinated Waters) published in The Handbook of Environmental Chemistry by Springer Berlin Heidelberg, 20014 (DOI: 10.1007/698_2014_263). This chapter is an introduction of all the present work. The references of this chapter are at the final of this part.

- Chapter III: Study of the transformation of two salicylates used in personal care products in chlorinated water, a paper (DOI: 10.1016/j.watres.2014.07.018) published with the results about the chlorination of two UV-filters: BzS and PS. This chapter is subdivided in Introduction, Material and Methods, Results and Discussion and References.
- Chapter IV: Study of the transformation of 4-MBC and OC in chlorinated water and by UV radiation. Here, the degradation of these UV-filters by chlorine and UV-radiation is assessed. This chapter is subdivided in Introduction, Material and Methods, Results and Discussion and References
- Chapter V: Toxicological assays – A first approach, where the toxic effects of another UV-filter (BDM) and its DBPs is assessed in larvae of zebrafish (*Danio rerio*). This chapter is subdivided in Introduction, Material and Methods, Results and Discussion and References
- Chapter VI: Conclusions, where are the conclusions of all the results of the present work.

1.4. References

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Chapter II

State of art

2.1. UV-filters chlorination

The following chapter was written in response to the invitation from Springer International Publishing Switzerland to the group of Professor Joaquim Carlos Gomes Esteves da Silva. The design of the structure and the selection of the topics covered were conducted by the three authors of the publication according to the publisher suggestion to address the topic of “occurrence of personal care products and transformation process in chlorinated waters”. The text was written by the author Mariana M. de Oliveira e Sá. The supervision, revisions and suggestions of improvement were added by Margarida S. Miranda and Joaquim C. G. Esteves da Silva.

Metadata of the chapter that will be visualized online

Chapter Title	Occurrence of Personal Care Products and Transformation Processes in Chlorinated Waters	
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Copyright Holder	Springer International Publishing Switzerland	
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Abstract	Personal care products (PCPs) have been found in surface water, wastewater, tap water, and swimming pool water. The chlorine used in the disinfection process of water reacts with these compounds generating chlorinated byproducts that may possess enhanced toxicity.	

In the case of swimming pool water chlorine also reacts with organic material released by swimmers such as amino acids and other nitrogen compounds yielding chlorinated compounds. Besides this organic material, sunscreen cosmetics used by swimmers are also released into pool water and react with chlorine. UV-Filters 2-ethylhexyl-*p*-dimethylaminobenzoate (EHDPABA), benzophenone-3 (BP-3), benzophenone-4 (BP-4), 2-ethylhexyl-4-methoxycinnamate (EHMC), and 4-methoxy-4′-*tert*-butyldibenzoylmethane (BDM) are known to suffer an electrophilic aromatic substitution of one or two atoms of hydrogen per one or two chlorine atoms leading to mono- and di-chlorinated byproducts. It has also been observed the presence of halobenzoquinones (HBQs) in pool water that results from the chlorination of UV-filters such as BDM, octocrylene, and terephthalilidene dicamphor sulfonic acid. The chlorination of some parabens has also been studied. It is known that some of the formed chlorinated byproducts are genotoxic. In this chapter we present a review on the work done so far to determine the stability of PCPs in chlorinated water and to identify the chlorinated byproducts.

Keywords (separated by "-")	Chlorinated byproducts - Chlorination - Personal care products - UV-filters

Occurrence of Personal Care Products and Transformation Processes in Chlorinated Waters

Mariana M. de Oliveira e Sá, Margarida S. Miranda, and Joaquim C.G. Esteves da Silva

Abstract Personal care products (PCPs) have been found in surface water, wastewater, tap water, and swimming pool water. The chlorine used in the disinfection process of water reacts with these compounds generating chlorinated byproducts that may possess enhanced toxicity.

In the case of swimming pool water chlorine also reacts with organic material released by swimmers such as amino acids and other nitrogen compounds yielding chlorinated compounds. Besides this organic material, sunscreen cosmetics used by swimmers are also released into pool water and react with chlorine. UV-Filters 2-ethylhexyl-*p*-dimethylaminobenzoate (EHDPABA), benzophenone-3 (BP-3), benzophenone-4 (BP-4), 2-ethylhexyl-4-methoxycinnamate (EHMC), and 4-methoxy-4′-*tert*-butyldibenzoylmethane (BDM) are known to suffer an electrophilic aromatic substitution of one or two atoms of hydrogen per one or two chlorine atoms leading to mono- and di-chlorinated byproducts. It has also been observed the presence of halobenzoquinones (HBQs) in pool water that results from the chlorination of UV-filters such as BDM, octocrylene, and terephthalilidene dicamphor sulfonic acid. The chlorination of some parabens has also been studied. It is known that some of the formed chlorinated byproducts are genotoxic. In this chapter we present a review on the work done so far to determine the stability of PCPs in chlorinated water and to identify the chlorinated byproducts.

Keywords Chlorinated byproducts, Chlorination, Personal care products, UV-filters

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M.S. Diaz-Cruz and D. Barceló (eds.) *Personal Care Products in the Aquatic Environment*, Hdb Env Chem, DOI 10.1007/698_2014_263,

Contents

1 Introduction

Personal care products (PCPs) have been found in surface water such as lakes, rivers, and sea, wastewater, and tap water [1–4]. The main reason for this is that during the wastewater treatment, the parental compounds are not totally removed and, in several cases, they also suffer biodegradation and biotransformation [5]. Then, the release of these effluents in the environment leads to the occurrence of PCPs and derivatives in the locations above mentioned. PCPs have been also found in bathing waters and swimming pool water due to their use by swimmers [6] by washing bath effect during bathing and swimming activities [7]. The problem is that, as in drinking water, the chlorine used in the disinfection process reacts with these compounds generating chlorinated byproducts that may possess enhanced toxicity [6, 8, 9]. Also body fluids such as urine and sweat mainly constituted by organic compounds can act as disinfection byproducts (DBPs) precursors [10]. Urea, amino acids, uric acid, gluconic acid, and sodium chloride are the major components of urine and sweat released by swimmers [11, 12]. However, waters disinfection is essential to kill microbial pathogens [13] that are mostly introduced into the water by humans [6].

In this chapter we present a review of reports on the chlorination of PCPs.

2 Reaction with Chlorine

2.1 Chlorination of Organic Matter Present in Body Fluids

In 2007, Li and Blatchley III [14] conducted a study to identify DBPs that result from chlorination of organic-nitrogen compounds present in pool waters due to urine and sweat released from human body. For instance, they verified that urea, creatinine, L-histidine, and L-arginine are trichloramine precursors. A few years later, Kanan and Karanfil [15] observed that some amino acids in urine, such as histidine and aspartic acid, are responsible for high formation rates of haloacetic acids (HAA), and that citric acid present both in urine and sweat is a chloroform precursor, just like albumin. All these information is compiled in Table 1.

Table 1 Disinfectant byproducts (DBPs) and corresponding precursors present in body fluids

DBP	Precursor	Body fluid
Haloacetic acids	Aspartic acid	Urine
	Histidine	Urine
Chloroform	Albumin	Urine, sweat
	Citric acid	Urine, sweat
	Creatinine	Urine, sweat
	Urea	Urine
	Glucuronic acid	Urine
	Hippuric acid	Urine
	Lactic acid	Urine
	Uric acid	Urine
Trichloramine	Creatinine	Urine, sweat
	L-histidine	Sweat
	L-arginine	Sweat
	Urea	Urine, sweat
Dichloromethylamine	Creatinine	Urine, sweat
Dichloroacetonitrile	L-histidine	Sweat

Based on [14, 15]

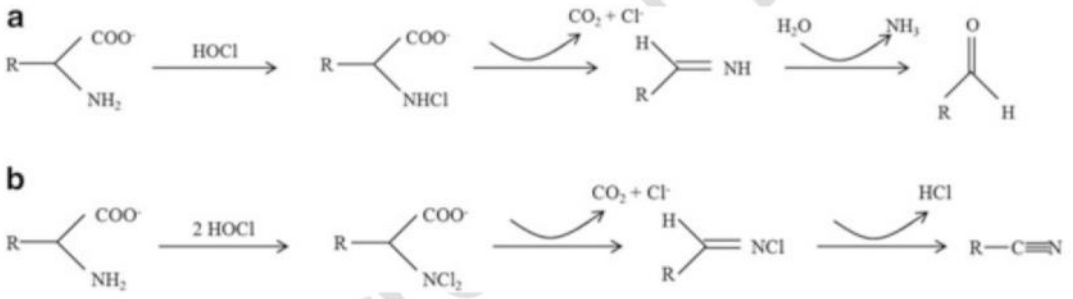

Fig. 1 Amino acid chlorination depending on chlorine dose: (**a**) formation of monochloramines due to the reaction with one HOCl molecule and (**b**) formation of dichloroamines due to the reaction with two HOCl molecules. Adapted from [13, 16]

Concerning amino acids chlorination, it begins with organic mono- or dichloramines formation which depends on chlorine dose and is followed by carbonyl or nitrile compounds production through decarboxylation and deamination (Fig. 1).

The chlorination of body fluids and other compounds is regulated by several factors. The presence of ion bromide (Br^-) influences the levels of halogenated DBPs increasing them, because it is more reactive than chlorine in HAA formation. Although its contribution for DBPs formation is complicated and without a defined pattern, the pH also interferes in this reaction. In some situations, such as nitrile formation, low pH acts favoring the DBPs formation [16] but, in another cases, it does exactly the opposite [13]. Water temperature, total organic content, and number of people in the water [6], dose and residual disinfectant available in the water and contact time between reactants [7] also impact DBPs formation.

2.2 Chlorination of Personal Care Products

On the other hand, pool water also contains PCPs. Inside this category are cosmetic ingredients, food supplements and other products like shampoos, lotions, and sunscreens cosmetics [17]. Sunscreens cosmetics are any cosmetic which contains a UV filter in its formulation to protect human skin from the solar UV radiation since they absorb, reflect and/or scatter UV radiation with a wavelength between 320 and 400 nm for UVA and between 290 and 320 for UVB [7, 18, 19]. There are two types of UV-filters: the organic (or chemical) and the inorganic (or physical) [19]. Inorganic UV-filters category only contains titanium dioxide (TiO_2) and zinc oxide (ZnO), which are known to reflect and scatter UV radiation. Regarding organic UV-filters, there are several classes such as *para*-amino-benzoates, cinnamates, benzophenones, dibenzoylmethanes, camphor derivates, and benzimidazoles and these compounds absorb the UV radiation [7]. There are many UV-filters allowed for use but their maximum concentration depends on legislation. Although European legislation differs from other countries legislation, like the USA and Japan, the usual concentration of UV-filters in cosmetics is between 0.1 and 10% [19].

Most of the organic UV-filters are relatively lipophilic and their structures contain aromatic rings, conjugated with carbon–carbon double bonds [18] and one benzenic moiety (or more) which has an efficient electronic delocalization due to the conjugation with electron releasing and electron acceptors groups located in either *ortho* or *para* positions. It is this feature that provides a specific maximum absorbance wavelength to the UV-filters [7].

UV-filters are known to react with chlorine leading to halomethanes, such as chloroform, haloacids, halonitriles, haloaldeydes, haloketones, halonitromethanes, haloamines, haloamides, and haloalcohols [17, 20] and also chlorinated UV-filter structures [18].

2.2.1 UV-Filters Chlorination

Few papers have been published in order to study both the UV-filters stability in chlorinated waters and to identify the resulting DBPs. In Fig. 2 we represent the UV-filters whose chlorination reaction was already studied.

In 2008, Negreira and co-workers [18] performed a study to assess the reactivity of three UV-filters containing hydroxy or amino groups in chlorinated waters: 2-ethylhexyl salicylate (ES), 2-ethylhexyl-*p*-dimethylaminobenzoate (EHDPABA), and benzophenone-3 (BP3). They found that the stability of these UV-filters is related with the pH: EHDPABA is more stable at basic water and for BP3 it happens exactly the opposite. ES showed a high stability independent of pH whereby ES halogenated reactions were considered negligible in real-life situations, since in this case there are several organic species competing for available chlorine. The following order of stability for these UV-filters was observed to be: BP3<EHDPABA<ES. However,

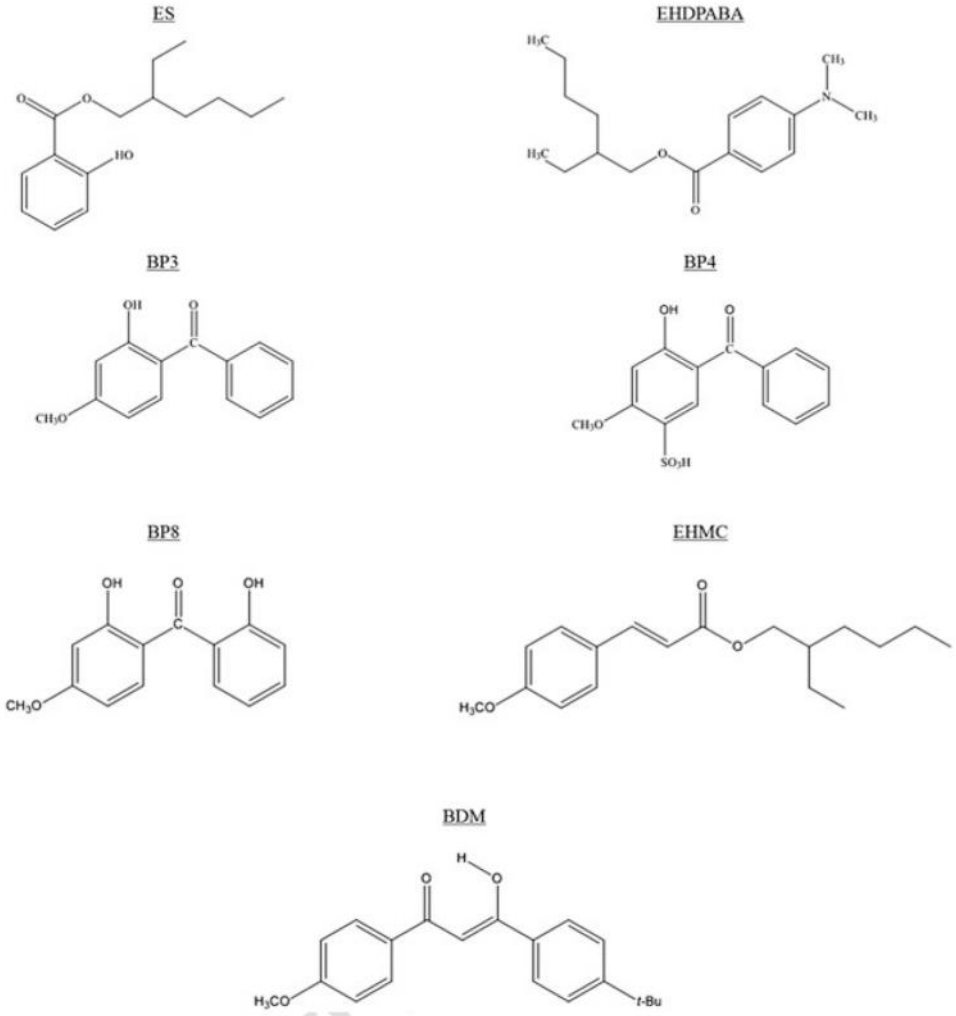

Fig. 2 Chemical structure of 2-ethylhexyl salicylate (ES), 2-ethylhexyl-*p*-dimethylamino-benzoate (EHDPABA), benzophenone-3 (BP3), benzophenone-4 (BP4), benzophenone-8 (BP8), 2-ethylhexyl-4-methoxycinnamate (EHMC) and 4-*tert*-butyl-4′-methoxydibenzoylmethane (BDM)

it was verified that bromide addition, even at low concentrations, reduces the UV-filters stability, especially for EHDPABA. This occurs due to bromide formation which largely reacts with aromatic compounds. Thus, differences among stabilities show the effect of different organic groups on the activation or deactivation of the phenolic ring towards electrophilic substitution reactions [7].

About DBPs, Negreira et al. [18] observed the formation of mono-halogenated species resulting from EHDPABA chlorination and the formation of mono- and di-substituted byproducts from BP3. These DBPs are formed by hydrogen replacement per chlorine in the aromatic rings. Although it is not demonstrated, looking at the parent species structure and considering the activation effects of the hydroxyl and amino groups towards electrophilic substitution reactions, it can be assumed

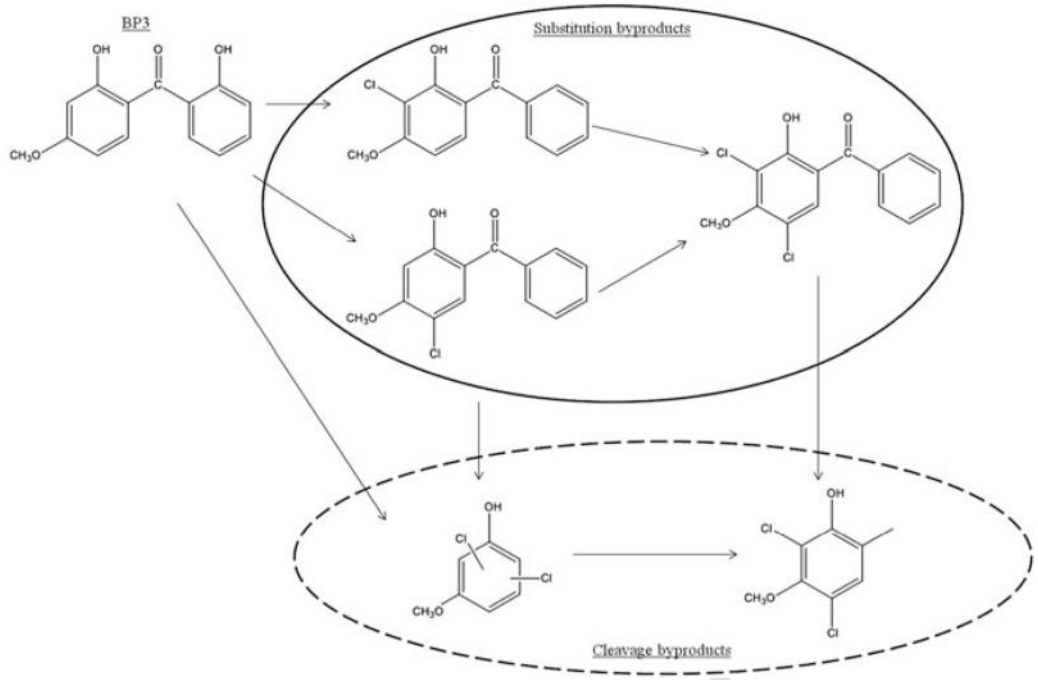

Fig. 3 Degradation pathway for BP3 proposed by Negreira et al. [18]

that these replacements occurred at the carbons in *ortho*- to the amino moiety (EHDPABA) and in *ortho*- and *para*- to the hydroxyl group (BP3).

Summarizing, EHDPABA has a relatively simple degradation pathway and the same pattern was also verified for BP3 which resulted in mono- and dihalogenated byproducts: Cl-BP3 (2 isomers) and Cl_2-BP3 (1 isomer). However, in the case of BP3, another group of byproducts was detected. Negreira et al. [18] identified halogenated forms of 3-methoxyphenol generated from cleavage of the carbonyl bond between the two aromatic rings in the BP3 molecule followed by methoxyphenol fragment halogenation. Moreover, mono- and dihalogenated BP3 substitution byproducts might also break down rendering different halogenated methoxyphenols. Figure 3 represents the reaction pathway for BP3 proposed by Negreira et al. [18]. All the DBPs of EHDPABA and BP3 showed a considerable stability.

The degradation of EHDPABA was previously studied by Sakkas et al. [21] in distilled, sea, and swimming pool water and the authors found one dichlorinated byproduct of the UV-filter and also mono- and dichlorinated degradation products of EHDPABA.

BP3 belongs to the benzophenones class of UV-filters approved by European legislation, which contains only another filter: benzophenone-4 (BP4) (Fig. 2). The stability of BP4 and its chlorination as well as its DBPs were also determined by Negreira et al. [22]. BP4 shows a low stability which decreases even more with pH increasing. As it happens with BP3, bromide addition decreases BP4 stability for the same reason of the first one.

The reaction between BP4 ($C_{14}H_{12}O_6S$) and chlorine yields three DBPs designated as B1 ($C_{14}H_{11}O_6SCl$), B2 ($C_{14}H_{11}O_7SCl$), and B3 ($C_{14}H_{10}O_7SCl_2$) by Negreira et al. [22]. B1 results from an electrophilic substitution of hydrogen per chlorine and this reaction is similar to the BP3 chlorination described above. The difference between B1 and B2 is one atom of oxygen which occurs due to the oxidation of the carbonyl group to an ester moiety (known as the Baeyer–Villiger reaction) with loss of a benzoyl moiety and ester bond established between the carbonyl group and the BP4 phenolic ring. Regarding B3, a dichlorinated byproduct, it is formed when B2 suffers electrophilic substitution of hydrogen per chlorine in carbon number 6 of the phenolic ring. Although the presence of hydroxyl- and methoxyl-functionalities in carbons located in meta-position deactivates this type of reaction, there exists an atom of oxygen in *ortho-* to carbon number 6 due to the Baeyer–Villiger reaction, which increases the probability of a electrophilic attack by chlorine [22].

Re-evaluating the BP3 chlorination with the methodology used in BP4 studies, Negreira [22] observed two other BP3 byproducts which had empirical formula $C_{14}H_{10}Cl_2O_4$ and $C_{14}H_9Cl_3O_4$. The first one is formed when the UV-filter undergoes its most important reaction pathway: two successive electrophilic substitutions of hydrogen per chlorine in carbons located at positions number 3 and 5 in the phenolic ring [18] but only when chlorine level is 0.03 μg/mL and at long reactions [22]. However, this byproduct is also compatible with oxidation of the carbonyl bridge in the molecule of BP3 to an ester group but only after the first reaction. The second byproduct ($C_{14}H_9Cl_3O_4$) appears due to further electrophilic substitution of hydrogen per chlorine in carbon number 6 of the $C_{14}H_{10}Cl_2O_4$ at chlorine concentrations above 2 μg/mL [22].

So, it can be said that the most favorable reaction pathway of both BP3 and BP4 with free chlorine consists of electrophilic substitutions of hydrogen per chlorine in carbon numbers 3 and 5 (*ortho-* and *para-* to the 2-hydroxyl moiety). Only after this reaction or when these carbons are already attached to other functionalities, the carbonyl group is converted into an ester moiety which links the two aromatic rings of these UV-filters. Finally, the aromatic ring bonded to the atom of oxygen in the ester group might undergo a further electrophilic substitution reaction [22]. Figure 4 represents the reaction pathway of this BP4 with free chlorine proposed by Negreira et al. [22].

Chloroform was also found as stable byproduct resulted from chlorination of BP3 and another benzophenone: benzophenone-8 (BP8) (Fig. 2) [20]. Chloroform formation is a function of pH and occurs in the presence of excess chlorine. However, BP3 and BP8 exhibited different chloroform formation behavior depending on pH: for the first one, chloroform formation decreases when pH increases from 6 to 10. This behavior is generally not only due to the speciation of aqueous chlorine (HOCl to Cl^-) but also due to the speciation of BP3 to the phenolate form, since chloroform/phenol molar yields have pH 8 as average for phenols and substituted phenols. Therefore, there is less HOCl to react with BP3. Concerning BP8, chloroform formation increases as pH increases from 6 to 10, probably due to 3-methoxy and the *ortho-* substituted phenolic moieties in

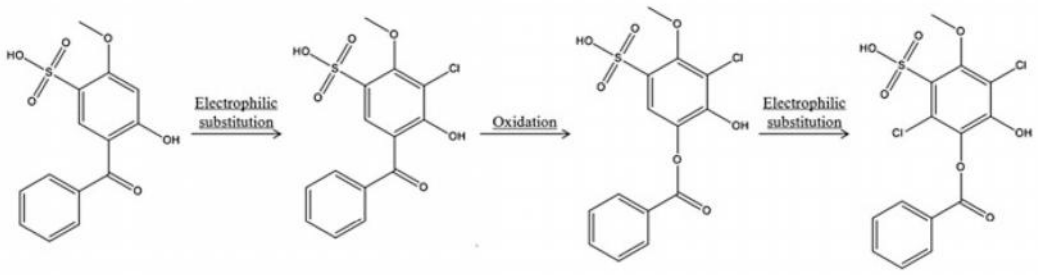

Fig. 4 Chlorination reaction for BP4 proposed by Negreira et al. [22]

BP8 molecular structure being less reactive with aqueous chlorine than BP3. Despite all of this, 3-methoxyphenol moiety appears to be the primary function group responsible for chloroform formation for both UV-filters [20].

There are two other UV-filters which are typically together in many commercial sunscreens: 2-ethylhexyl-4-methoxycinnamate (EHMC) and 4-*tert*-butyl-4′-methoxydibenzoylmethane (BDM). The first one has absorption capacity in the UVB range and the second one in UVA. Therefore, these two UV-filters combined offer UV protection over a wider range of wavelengths. Although EHMC and BDM are present in sunscreens as the isomer *E* for the first one and as enol form for the second one, under irradiation EHMC suffers isomerization from *E* to *Z* form (Fig. 5a) and BDM tautomerizes from enol to keto form (Fig. 5b) [7].

Santos [23] observed six byproducts resulted from EHMC chlorination: two of them are dichlorinated products ($C_{18}H_{24}O_3Cl_2$) and the rest of them are comprised of monochlorinated byproducts ($C_{18}H_{25}O_3Cl$). Both types of byproducts are probably the result of hydrogen replacement by chlorine in the benzene ring of EHMC in the same way already described above. Regarding BDM byproducts it was observed one monochlorinated byproduct ($C_{20}H_{21}O_3Cl$) and one dichlorinated ($C_{20}H_{20}O_3Cl_2$). However, a similar reaction pattern is observed for these two UV-filters because the substitution of hydrogen atoms by chlorine can only occur in the benzene ring containing methoxy group, since chlorination in the benzene ring containing the *t*-Bu group is highly prohibitive due to the large volume of this group.

The reaction between chlorine and each of these UV-filters is regulated by some factors, such as pH, chlorine concentration, temperature, dissolved organic matter (DOM), and irradiation time. The principal factor affecting the EHMC chemical transformation is pH since the lower is the pH, the higher is the transformation percentages of EHMC. The explanation for this fact is that the main chlorine species present at low pH is HOCl (in contrast with at higher pH, where the hypochlorite anion (OCl^-) is prevalent) which is more reactive towards EHMC, resulting in higher degradation. Nevertheless, higher temperature values also lead to higher transformation percentages and this is almost independent of the pH. Concerning BDM, chlorine concentration is the principal factor affecting its transformation percentage, since higher concentrations of chlorine will favor chlorine attack and the incorporation of chlorine in the UV filter structure even at high

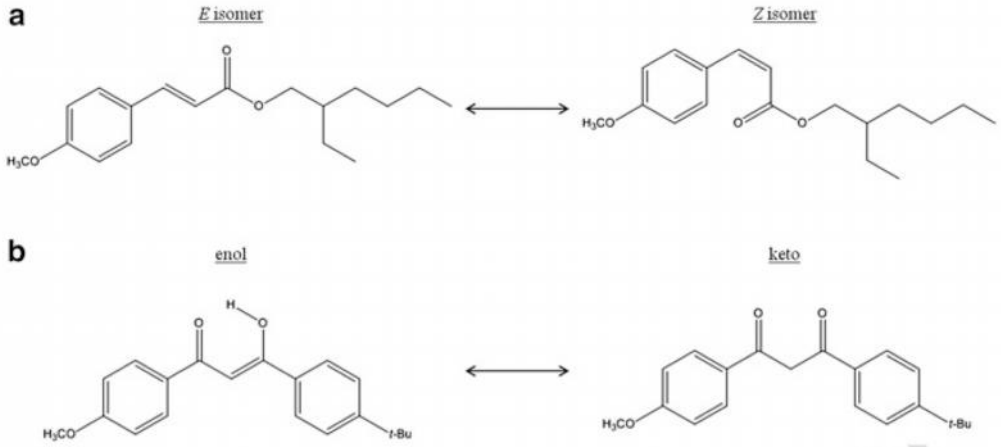

Fig. 5 (**a**) Photoisomerization of the UV-filter 2-ethylhexyl-4-methoxycinnamate (EHMC); (**b**) tautomerism of the UV-filter 4-*tert*-butyl-4′-methoxydibenzoylmethane (BDM)

pH values. However, in presence of DOM, transformation percentages of BDM are low probably due a competition process between the UV-filter and DOM for the available chlorine [23].

Halobenzoquinones Formation

It was also observed the presence of halobenzoquinones (HBQs) in pool water resulted from sunscreens chlorination. Aromatic structures in these PCPs such as phenols and quinones are likely to be the precursors of HBQs as well as some common ingredients of lotions, like benzyl alcohol, lecithin, parabens, and fragrances. UV-filters such as avobenzone, octocrylene (2-ethylhexyl-2-cyano-3,3-diphenyl-2-propenoate, OCT), and terephthalilidene dicamphor sulfonic acid may also be HBQ precursors [24]. Wang et al. [24] observed the formation of 2,6-dichloro-1,4-benzoquinone from the reaction between chlorine and four sunscreens containing organic and inorganic UV-filters. Although warm water provides a comfortable environment for swimmers, this fact may accelerate the chlorination reaction to produce more HBQs [24].

Besides 2,6-dichloro-1,4-benzoquinone, 2,6-dichloro-3-methyl-1,4-benzo-quinone, 2,3,6-trichloro-1,4-benzoquinone, and 2,6-di-bromo-1,4-benzoquinone also are common DBPs in chlorinated water [25].

2.2.2 Parabens Chlorination

Besides sunscreens, other PCPs such as parabens may also be present in pool water. Parabens belong to a group of bactericides and preservative agents in PCPs and they are continuously released in aquatic media through domestic wastewater and, although they are almost completely removed during sewage water treatments,

they have been detected in rivers at low ng L^{-1} level. Considering the extensive employment of the compounds in PCPs, activities like showering and bathing constitute a source of dermal exposition to parabens DBPs [26]. The potential degradation of four alkylated parabens (methyl, ethyl, propyl, and butyl paraben) and the formation of DBPs were investigated by Canosa et al. [26]. Five transformation species were detected for each parent paraben corresponding to mono- and dichlorinated compound. Similar to some UV-filters, they are formed by a substitution of one or two atoms of hydrogen per chlorine in the aromatic ring and this chlorination occurs in both carbons in *ortho-* to the phenolic group, since the *para-* position is blocked with the ester moiety. In tap water, the chlorine content is usually enough to produce significant amounts of these DBPs in few minutes. However, the dichlorinated byproducts are rather resistant to undergo further chlorine substitution reactions or cleavage of the aromatic ring, even in presence of relatively high concentrations of chlorine. So, if they are generated in real-life situation, their presence in the aquatic environment is feasible [26].

3 Toxic Effects of UV-Filters and Its Chlorination Byproducts

It is known that byproducts formed from reaction between chlorine and natural organic matter of water, such as chloroform as well other trihalomethanes, nitrosamines, haloacetic acids, etc., have toxic effects like carcinogenic effects in animals and human beings [27]. Now, it is mandatory to assess the toxicity of DBPs formed from PCPs chlorination. The knowledge of this subject is still poor but there are already a few papers published in order to study the toxicity of some of these compounds.

Bladder cancer has been associated with exposure to chlorination byproducts in drinking water, and experimental evidence suggests that exposure also occurs through inhalation and dermal absorption during swimming in pools because certain DBPs have high volatility and dermal permeability. Villanueva et al. [28] observed that subjects who had ever swum in a pool showed an increased risk of bladder cancer compared with those who had never swum in pools and former and current smokers present an excess risk of bladder cancer. This study also revealed a duration-response relation for cumulative time spent in swimming pools. To evaluate the genotoxicity of swimming pool water in swimmers, Kogevinas and co-workers [29] examined some biomarkers of genotoxicity in an experimental study in which adults swam for 40 min in a chlorinated, indoor swimming pool, comparing the biomarker results with the concentrations of four THMs (bromoform, bromodichloromethane, chloroform, and chlorodibromomethane) in exhaled breath. It was observed increases in two biomarkers of genotoxicity (micronuclei in peripheral blood lymphocytes and urinary mutagenicity). Although only brominated THMs showed genotoxicity, all four are carcinogenic in rodents.

It was also verified that recreational pool waters are more genotoxic [30] and cytotoxic than tap water and this elevated genotoxicity and cytotoxicity are associated with many classes of nitrogenous-DBPs (N-DBPs) [10]. The higher genotoxicity of the recreational pools compared to the tap water source could reflect prolonged disinfectant contact times [30].

Furniture conditions, such as illuminations condition, also affect the cytotoxicity of pool water [10, 30]: The pool water under indoor conditions was more cytotoxic ($LC_{50} = 24.2\times$) than when it was operated as an outdoor pool ($LC_{50} = 181.4\times$). The outdoor pool exposed to sunlight featured lower cytotoxicity than the same pool under indoor conditions which indicate that either the compounds responsible for the cytotoxicity, or their precursors, may be photolabile [10] or have increased volatilization [30]. Physical activity appears to enhance the absorption of DBPs [31].

UV-filters have high lipophilicity (mostly with $\log K_{ow}$ 4–8) whereby they have been shown to accumulate in the food chain and in human milk fat. However, at present, there is a scarcity of data on environmental concentrations of UV-filters [32, 33]. Moreover, concentrations reported fluctuate significantly as a function of sample location, size of the system under study (e.g., lakes and swimming pools), frequency and type of recreational activities, season of the year, and hour of the day. Still, maximum concentrations reported have corresponded to mid-day on warm summer days, as expected [33]. Among UV-filters, octocrylene is of great concern since it has a high lipophilicity (K_{ow} 6.88). Actually, this UV-filter has already detected in liver tissues of dolphins (*Pontoporia blainvillei*) with concentrations in the range 89–782 ng/g/lw and there is evidence that maternal transfer may occur trough placenta and likely also through breast milk [34].

4 Conclusions and Further Researches

Disinfection of drinking water is important for public health but many people are exposed to chlorination byproducts not only through ingestion but also through other activities such as showering, bathing, and swimming [35]. So, future studies should evaluate more completely the uptake and potential effects of a range of DBPs present in pool water [29]. Although the mixture of the byproducts may differ by geographic area and time, studies are needed to examine the potential effects of these mixtures [35]. Furthermore, it is important to examine the various exposure pathways and routes other than ingestion in more detail.

Reports on the occurrence of sunscreen agents in natural waters have so far been scarce and have mainly focused on bathing waters in closed systems (e.g., swimming pools or small lakes). A great deal of additional data is needed to understand the significance of UV-filters in the aquatic environment. It is also necessary to increase knowledge of their bioaccumulation in humans and wildlife [33]. It is also important that further researches take into account pool operation/maintenance. Pool disinfection is essential to preventing exposure to pathogens; still, DBP

formation can be reduced with proper disinfectant use along with known engineering solutions. Unhygienic practices enhance the amount of organic matter released by swimmers through urine and other body fluids. So, substantial investments into education and outreach will be necessary to affect these behaviors and practices. By improving disinfection practices and reducing the input of contaminants both chemical and biological, the goal of healthier pools and healthier people can be achieved [6]. For example, showering and using toilet facilities, washing off sunscreen lotions, and applying water-tight diapers can reduce the bather load and help to reduce the potential for DBP formation [36]. If swimmers take showers frequently, DBPs will be removed on skin preventing them from deeper penetration [37].

Environmental chemistry studies should also focus on strategies to minimize the formation of chlorinated byproducts of UV-filters by the development of new sunscreen formulations that prevent the release of UV-filters into chlorinated water [23].

Haloquinones have been proving to be more toxic than the regulated halomethanes [25]. The potential toxic effects of these compounds warrant further investigations into the occurrence, human exposure, and management of haloquinones in chlorinated water [25].

Regarding other cosmetics ingredients further studies are needed to evaluate potential human health risks and ecotoxicological effects of halogenated byproducts and to know their fate in the environment [26, 27].

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2.2. Pharmaceuticals chlorination

Besides sunscreens, others PPCPs such as pharmaceuticals may also be present in pool water.

For instance, the antimicrobial triclosan is used in hygienic products like soaps and bodywash and can therefore be released into water during swimming activities. When it comes into contact with chlorine, triclosan suffers chlorination yielding several byproducts (Fiss *et al*., 2007). Chloroform and chlorophenol are the two DBPs formed from triclosan chlorination, however they appear only under specific conditions. The chlorophenol yields are inversely correlated with the chloroform yields. Fiss *et al*. (Fiss *et al*., 2007) observed that in unfavorable conditions for chloroform formation, in addition to chlorophenol it is also released intermediate (chlorophenoxy)phenols: 5,6-dichloro-2-(2,4-dichlorophenoxy)phenol, 4,5-dichloro-2-(2,4-dichlorophenoxy)phenol and 4,5,6-trichloro-2-(2,4-dichlorophenoxy)phenol). This happens maybe because chloramines are formed due to the presence of another soups's ingredients, like ammonium salt. Those chloramines react with triclosan producing chlorophenols and (chlorophenoxy)phenols, but have insufficient oxidizing power to cleave the phenol ring of triclosan and produce chloroform. So, chloroform is only yielded when a sufficient excess of free chlorine is present to cleave the phenol ring of triclosan. Again, the soap composition may affect the amount of free chlorine because the other ingredients also suffer chlorination (Fiss *et al*., 2007).

Antipyrine (ANT) is a pharmaceutical and has anti-inflammatory and analgesics properties. It was already found in several aquatic environments because it cannot be removed completely during water and wastewater treatments (Cai *et al.*, 2013a; Cai *et al.*, 2013b). So, ANT appears in drinking water and, subsequently, in pool water. Then, reaction between chlorine and this pharmaceutical can occur. Cai *et al.* (Cai *et al.*, 2013b) identified sixteen chlorination by-products including a monochlorine substitution product (4-chloro-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one) called ANT-Cl, which results from halogenation by free chlorine attack (Fig.2.1) (Cai *et al.*, 2013a; Cai *et al.*, 2013b). The potential pathways of ANT chlorination were proposed, including halogenations, dealkylations and hydroxylations (Cai *et al.*, 2013b). But pharmaceutical chlorination is also regulated by some factors, such as initial chlorine concentration and pH. For instance, ANT chlorination reaction is faster when pH is lower than 7 (Cai *et al.*, 2013a) and increases with initial chlorine concentration increasing (Cai *et al.*, 2013b).

Cimetidine, another pharmaceutical found in the environment, reacts with chlorine (Fig.2.2) during water treatment yielding other DBPs which were already characterized. Given its structure, cimetidine chlorination may be expected to result in minor structural changes such as sulfur oxidation, electrophilic halogenation and N-chlorination of one or

more of its amino groups. Excess of free chlorine gives rise to cimetidine sulphoxide ($C_{10}H_{16}N_6SO$) and then to 4-hydroxymethyl-5-methyl-1H-imidazole which forms an ion - 5-methyl-4-methylene-1H-imidazol-1-ium – through loss of water (Fig.2.3). From this latter, it can be formed another intermediate - 4-chloro-5-methyl-1H-imidazole – but this requires the breaking of a C-C bound, a transformation not frequently observed in free chlorine reactions of PPCPs. Finally, it is formed a byproduct with chemical structure $C_5H_8N_4O_2S$ (Buth *et al.*, 2007). Buth *et al*. (Buth *et al.*, 2007) failed to distinguish by the mass spectrometric or spectroscopic data if this compound was a *β*-sultam, N-cyano-N'-methyl-N''-*β*-sultamylguanidine, or a *δ*-sultam, N-(2-methyl-1,1-dioxide-1,2,4-thiadiazinan-3-ylidene)cyanamide.

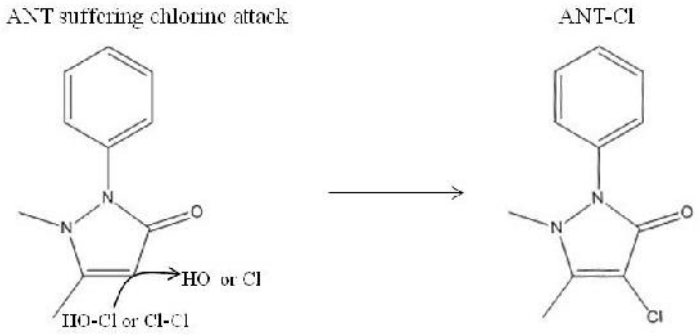

Figure 2.1. In the process of ANT chlorination, free chlorine can attack ANT to bring about halogenation. Adapted from Cai *et al.*, 2013a and Cai *et al.*, 2013b.

Cimetidine chlorination is also regulated by pH: the entire reaction pathway shown in Figure 7 with all its steps and intermediates may occur at acidic and neutral pH, whereas the direct conversion of cimetidine to 4-hydroxymethyl-5-methyl-1H-imidazole, 4-chloro-5-methyl-1H-imidazole and *β*-sultam, N-cyano-N'-methyl-N''-*β*-sultamylguanidine/*δ*-sultam, N-(2-methyl-1,1-dioxide-1,2,4-thiadiazinan-3-ylidene)cyanamide bypassing cimetidine sulfoxide, may occur under basic conditions (Buth *et al.*, 2007).

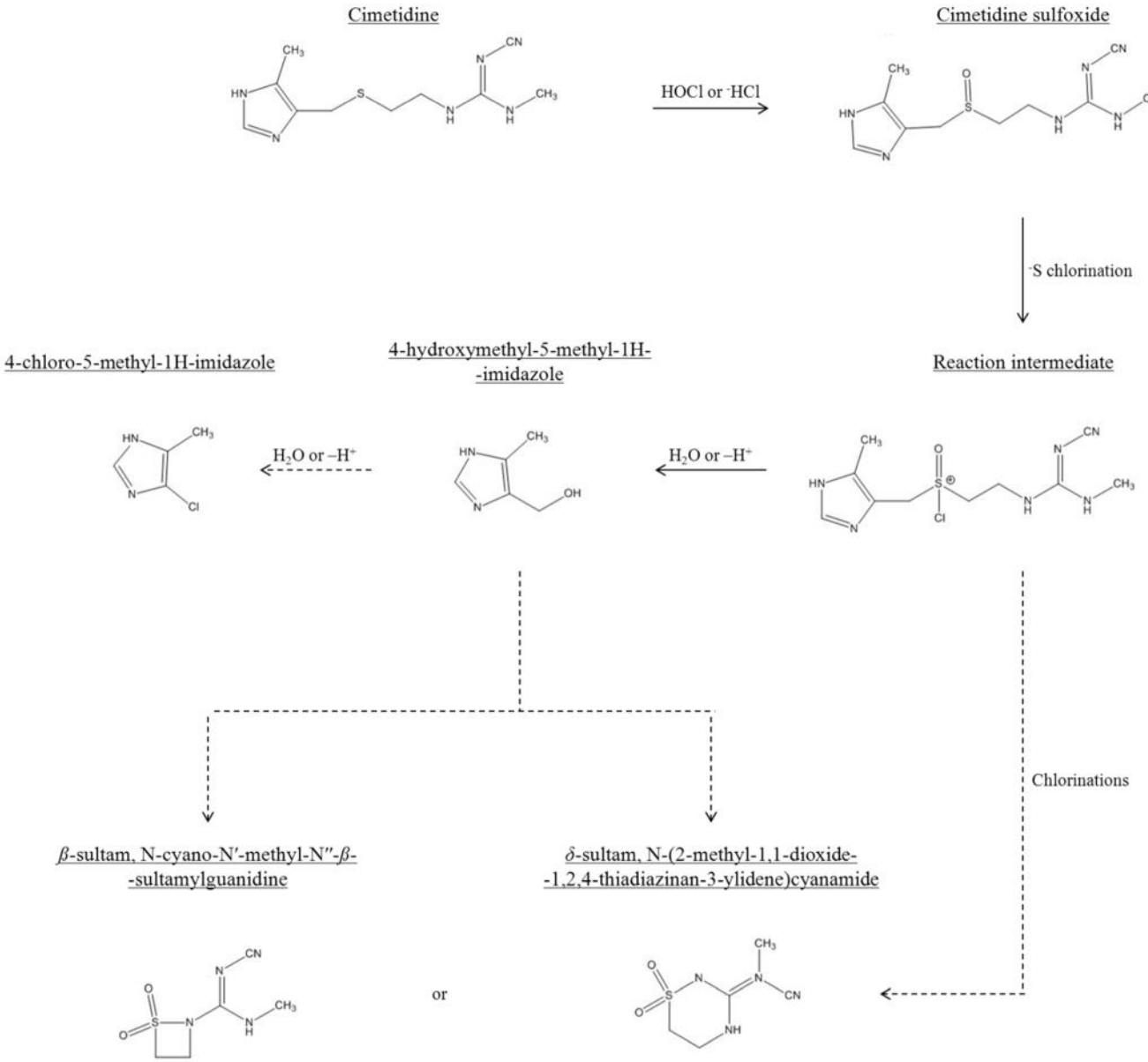

Figure 2.2. Reaction pathways for the cimetidine chlorination through the intermediacy of cimetidine sulfoxide proposed by Buth *et al.*, 2007)

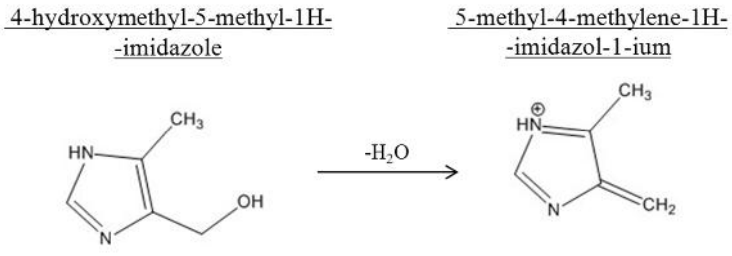

Figure 2.3. The detected ion 5-methyl-4-methylene-1H-imidazol-1-ium, resulting from the loss of water from 4-hydroxymethyl-5-methyl-1H-imidazole.

The chlorination of diclofenac, bezafibrate, salicylic acid, clofibric acid, naproxen, indomethacine, ketoprofen, ibuprofen and fenoprofen has also already been studied.

These pharmaceuticals are among the most frequently found in the environmental by the same reasons as above and they also suffer chlorination when they are present in tap water (Quintana *et al*., 2010). In 2010, Quintana *et al.* (Quintana *et al*., 2010) studied their degradation in chlorinated water but only salicylic acid, diclofenac, naproxen and indomethacine were further evaluated because they were the only ones that had degradation over 30%. In this study, it was verified that as the chlorine concentration increases, the reaction kinetics accelerates. Chlorination of salicylic acid yielded three DBPs due to halogenation in the ring activated positions 3 and 5: two isomers of chlorosalicylic (3-Cl-SA and 5-Cl-SA) acid and 3,5-dichlorosalicylic acid (Cl_2-SA). About chlorination of naproxen, chloronaproxen (Cl-naproxen) is formed. Diclofenac formed a monochlorinated byproduct (Cl-diclo) and another product (Cl-diclo-CO) which empirical formulae corresponding to the loss of a CO group from the first one. Cl-diclo-CO is probably formed through a lactone intermediate by decarboxylation and oxidation of the dichlorinated ring in position 4. In the case of Cl-diclo, it suffers losses of CO_2 and HCl. Regarding indomethacine, HOCl does not leads to halogenation but to oxidation as the monochlorination pattern of the deprotonated products is maintained. The major products from this reaction are two hydroxylated isomers (OH-indo) formed due to the losses of CO_2 and CO. Two decarboxylated hydroxylated products (OH-indo-CO_2) are also produced, as well as, two other minor products whose correspond to desahydro-indomethacine (indo-H_2) and 4-chlorobenzoic acid (Cl-BA), probably produced by the final hydrolysis of the amine group.

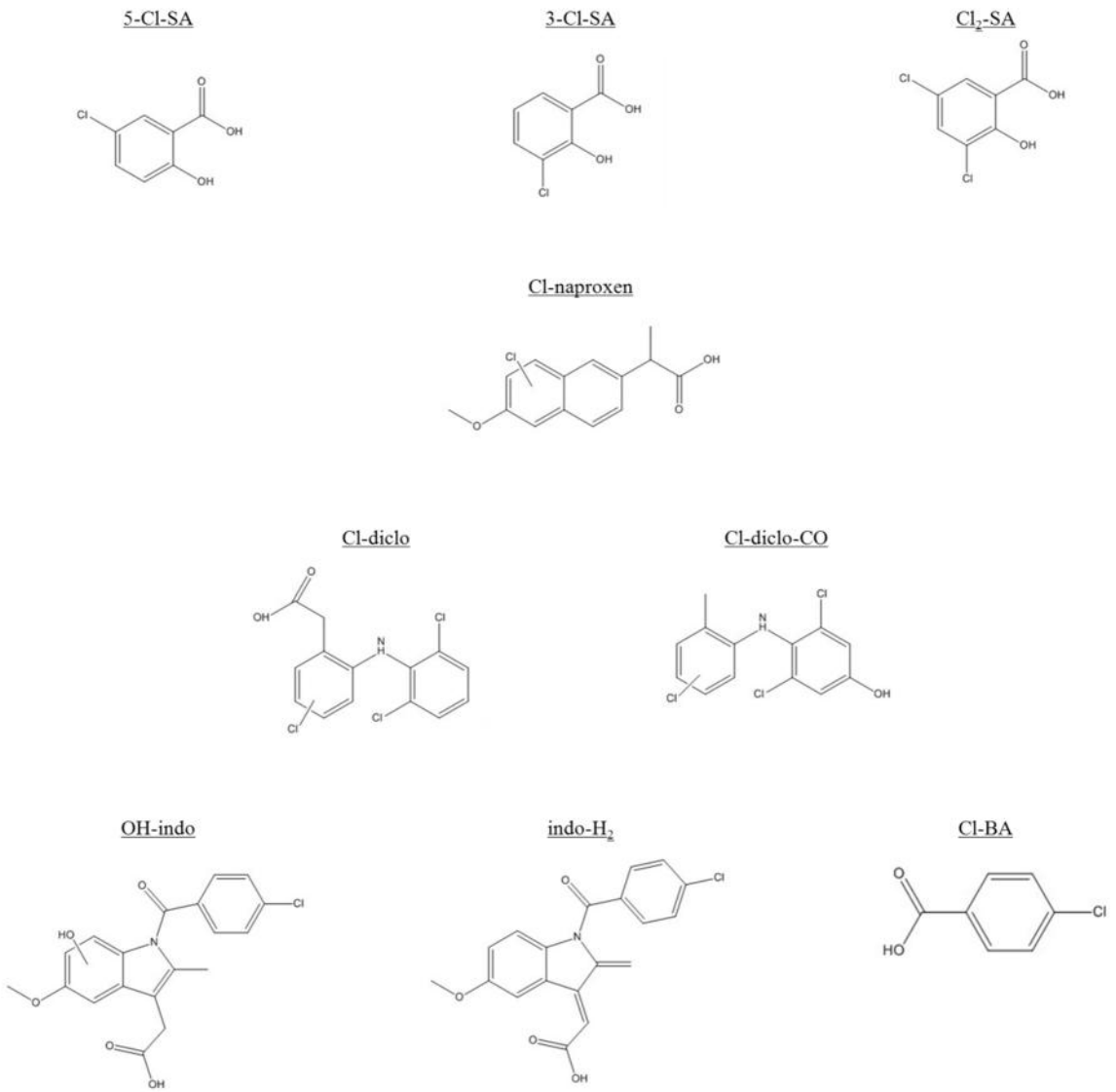

Figure 2.4. DBPs of salicylic acid, naproxen, diclofenac and indomethacine proposed by Canosa *et al.*, 2006

It is important to monitor the concentrations and the environmental behavior of these compounds. The complexity of the matrices and the fact that UV-filters are usually at trace concentrations levels require methods with enough sensitivity to report concentrations at the ng/L level. Methods based in liquid chromatography (LC) are a god separation technique although gas chromatography (GC) has recently been found as more powerful and faster. Mass spectrometry (MS) is the detection technique most frequently used and, due to the high absorbance of UV-filters in the UV range, UV/Vis spectrometry detector is useful, either with single-wavelength or with diode-array detection (DAD) attached to a LC system (Díaz-Cruz and Barceló, 2009).

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Chapter III

Study of the transformation of two salicylates used in personal care products in chlorinated water

The following article was published in Water Research Journal. The bibliographic research and all the experimental procedure were written and performed by Mariana M. de Oliveira e Sá. Revision of the manuscript, suggestions of improvement and correction were added by Margarida S. Miranda and Joaquim C. G. Esteves da Silva.

WATER RESEARCH XXX (2014) 1

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/watres

Graphical Abstract

Water Research 2014, ■, ■■■

Study of the transformation of two salicylates used in personal care products in chlorinated water

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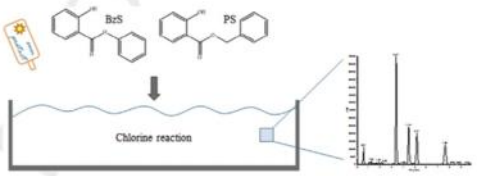

Highlights

- Salicylates used for sunscreen formulations react with free chlorine.
- Benzyl and phenyl salicylates have degradation half-times of a few hours in chlorinated water.
- Mono and dichloro salicylates are generated as disinfection byproducts.

http://dx.doi.org/10.1016/j.watres.2014.07.018
0043-1354/© 2014 Published by Elsevier Ltd.

WATER RESEARCH 65 (2014) 32–39

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Study of the transformation of two salicylates used in personal care products in chlorinated water

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ARTICLE INFO

Article history:
Received 20 February 2014
Received in revised form
5 June 2014
Accepted 14 July 2014
Available online 22 July 2014

Keywords:
Personal care products
Benzyl salicylate
Phenyl salicylate
Swimming pool
Disinfection by-products

ABSTRACT

Disinfection of swimming pool water is essential to inactivate pathogenic microorganisms. However chlorine based disinfectants, the most commonly used, are known to lead to the formation of disinfection by-products (DBPs), some of which have been associated with adverse health effects. Precursors of DBPs include the organic matter present in the water used to fill the swimming pool, human body fluids and personal care products (PCPs) used by swimmers and bathers. The increased use, in the last years, of PCPs lead to an increased concern about the fate of PCPs in swimming pool waters and potential health risks of formed DBPs. In this study, the chemical transformations of two salicylates, benzyl salicylate (BzS) and phenyl salicylate (PS), incorporated in several PCPs, in chlorinated water were investigated. High-performance liquid chromatography (HPLC) with UV-diode-array detection (HPLC-UV-DAD) was used to follow the reaction kinetics and HPLC with mass spectrometry (HPLC-MS) was used to tentatively identify the major transformation by-products. Under the experimental conditions used in this work both salicylates reacted with chlorine following pseudo-first order kinetics: rate constant $k = (0.0038 \pm 0.0002)$ min^{-1} and half-life $t_{1/2} = (182 \pm 10)$ min for BzS and rate constant $k = (0.0088 \pm 0.0005)$ min^{-1} and half-life $t_{1/2} = (79 \pm 4)$ min for PS (mean ± standard deviation). The reactions of the two salicylates in chlorinated water led to the formation of DBPs that were tentatively identified as mono- and dichloro- substituted compounds. Most probably they result from an electrophilic substitution of one or two hydrogen atoms in the phenolic ring of both salicylates by one or two chlorine atoms.

1. Introduction

Swimming and other pool water activities have significant positive health effects, related to the benefits of exercise and have some advantages over land-based activities for people of all ages and physical abilities. To conserve the positive aspects of swimming pool activities it is necessary to disinfect swimming pool water to protect swimmers and bathers from pathogenic microorganisms. However, disinfection has also

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http://dx.doi.org/10.1016/j.watres.2014.07.018
0043-1354/

unintended consequences. As with drinking water, chlorination is the most common disinfection method for swimming pool water. Chlorine based disinfectants, loosely referred as chlorine, react with the organic matter present in swimming pool water giving origin to a variety of halogenated organic compounds known as disinfection by-products (DBPs) (WHO, 2006; Zwiener et al., 2007; Lakind et al., 2010). Additionally to the precursors present in the water used to fill the pool, bathers/swimmers also introduce many compounds that come from urine, sweat, saliva, hair, skin cells and personal care products (PCPs) which include for e.g. lotions, cosmetics, and sunscreens (WHO, 2006). Currently more than one hundred DBPs have been identified in swimming pools including trihalomethanes, haloacids, halonitriles, haloaldeydes, haloketones, halonitromethanes, haloamines, haloamides and haloalcohols (Richardson et al., 2010) that are likely formed from precursors from body fluids, such as urine and sweat. Exposure to DBPs, by dermal contact and absorption through skin, inhalation and ingestion of water, has been linked to adverse health effects, like increased risk of asthma and other respiratory diseases as well as bladder cancer (Villanueva et al., 2007; Bernard et al., 2009). Although there is a great deal of information regarding the fate of urine and sweat constituents in swimming pool waters (Chowdhury et al., 2014), very little is known on organic compounds from PCPs (Santos et al., 2012). The occurrence of organic compounds from PCPs (UV-filters and anti-microbial agents) in swimming pool waters has been reported (Díaz-Cruz et al., 2008; Santos et al., 2012; Cuderman and Heath, 2007). The concentrations reported varied depending on the sampling location and the intensity of the recreational activities (Díaz-Cruz et al., 2008). Regarding PCPs fate in swimming pools there have been a few investigations on the reaction of UV filters, incorporated in sunscreens, in chlorinated waters. Ethylhexyl salicylate (Negreira et al., 2008), 2-ethylhexyl-p-dimethylaminobenzoate (Negreira et al., 2008; Nakajima et al., 2009; Sakkas et al., 2003), benzophenone-3 (Negreira et al., 2008; Zwiener et al., 2007), benzophenone-4 (Negreira et al., 2012), benzophenone-8 (Duirk et al., 2013), 2-ethylhexyl-4-methoxycinnamate (Santos et al., 2013; Nakajima et al., 2009; Zwiener et al., 2007), 4-*tert*-butyl-4′-methoxydibenzoylmethane (Santos et al., 2013), hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl]-benzoate (Grbovic et al., 2013), 2-ethylhexyl-2-cyano-3,3-diphenyl-2-propenoate (Zwiener et al., 2007), 2-phenyl-1H-benzimidazole-5-sulfonic acid (Zwiener et al., 2007) and 4-methylbenzylidene camphor (Zwiener et al., 2007) are the ones whose stability in chlorinated water was already studied and halogenated by-products were identified. Wang et al. (2013) also found that 4-*tert*-butyl-4′-methoxydibenzoylmethane, octocrylene, and terephthalilidene dicamphor sulfonic acid and common ingredients of lotions, such as benzyl alcohol, lecithin and parabens may be precursors of halobenzoquinones in swimming pool waters. The reaction of triclosan, a commonly used antimicrobial agent in hygienic products like soaps and bodywash, and chlorine was investigated and the by-products formed include chlorinated phenoxy-phenols, chlorinated phenols and trihalomethanes (Fiss et al., 2007). Parabens, extensively employed as bactericides and preservative agents in antiperspirants and sunscreens, were found to react with chlorine leading to paraben mono- and dichlorinated by-products (Canosa et al., 2006). Studies on the toxicity of halogenated PCPs are still largely lacking. The physicochemical properties (e.g., high lipophilicity) of PCPs and chlorinated derivatives can lead to high human exposure and their toxic effects cause human health concern.

In order to increase the knowledge about the fate of PCPs ingredients in swimming pool waters, the present study aims to assess the stability of two salicylates commonly used in PCPs: benzyl salicylate (BzS) and phenyl salicylate (PS) in chlorinated water, in conditions similar to those existent in swimming pools. The structural formulas of the two studied salicylates are presented in Fig. 1. Salicylates were one of the first class of UV-filters used in sunscreens (the first formulation was a combination of benzyl salicylate and benzyl cinnamate) (Palm and O'Donoghue, 2007). Nowadays, besides its utility as UV-filter, BzS is also used as flavor ingredient (Adams et al., 2005) and as fragrance and/or fragrance fixer in fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents (Charles et al., 2009; Lapczynski et al., 2007a). PS is currently used as an UV-filter (Jiménez-Díaz et al., 2013) and also as a fragrance (Lapczynski et al., 2007b) and plastic stabilizer due to its UV absorber capacity (Mohamed and Fahmi, 2007). It is known that BzS and PS may have toxic effects like antiestrogenic and antiandrogenic activities (González et al., 2008) as well cause oestrogenic responses in human breast cancer cells (Charles and Darbre, 2009). Animal and human scientific data relevant to the risk assessment of the use of salicylates, including BzS and PS, as fragrance ingredients has been reported (Belsito et al., 2007; Lapczynski et al., 2007a).

The aims of this work are: (i) to evaluate the stability of BzS and PS in water samples with different pH values and different free chlorine concentrations (ii) to determine their half-lives under specific experimental conditions; (iii)

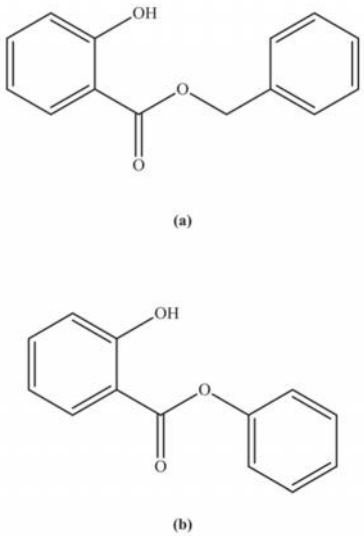

Fig. 1 – Structural formula of the two studied salicylates (a) BzS and (b) PS.

tentatively identify major DBPs and (iv) to study the stability of formed DBPs under time. The kinetics of the reactions between the two salicylates and chlorinated water and DBPs formation were studied by HPLC-UV-DAD. Before the identification of salicylates by-products by HPLC-MS a pre-concentration step was taken which involved a liquid–liquid extraction.

2. Materials and methods

2.1. Reagents

BzS, CAS Registry No. [118-58-1], 98%, (molecular formula $C_{14}H_{12}O_3$) and PS, CAS Registry No. [118-55-8], 99%, (molecular formula $C_{13}H_{10}O_3$) were purchased from Sigma–Aldrich. A commercial sodium hypochlorite solution with a chlorine content of <5% (28 mg/L of free chlorine) was used in the chlorination studies. This solution was stored at 4 °C and its free chlorine content was periodically measured by a small compact photometer for chlorine (HANNA Checker Mini Hi 701 series). Stock solutions of the salicylates were prepared with methanol from Merck. Deionized water (conductivity < 0.1 μS cm^{-1}) was used in all experiments. Ascorbic acid (99.7%) used to stop the chlorination reaction was obtained from Merck. The pH of the solutions was adjusted to a pre-determined value with HCl solutions 0.1 mol/L. Pre-concentration of the water samples for HPLC-MS analysis was performed by liquid–liquid extraction with ethyl acetate (analytical grade quality) purchased from Riedel-de Haën. Eluents for chromatographic analysis (methanol and acetonitrile) were liquid chromatographic grade and were bought from Merck.

2.2. Study of the effect of sample pH and free chlorine concentration

The concentration of free chlorine considered being acceptable in order to achieve adequate disinfection while minimizing users discomfort varies widely around the world. According to the World Health Organization (WHO, 2006) guidelines, the concentration of free chlorine in public and semi-public swimming pools should be maintained at 1–3 mg/L and in public and semi-public hot tubs at 2–5 mg/L and the pH should be maintained between 7.2 and 8.0.

In order to assess whether the two salicylates react to a significant extent with low chlorine concentrations, such as those used in swimming pools, a set of experiments was designed. Stock solutions of BzS and PS in methanol (about 100 mg/L) were first prepared. The reactions were carried out in glass vessels containing 100 mL of deionized water. The deionized water samples were spiked with free chlorine to get the initial concentrations of 1, 2, 3 and 5 mg/L. After that, the pH of each aqueous solution was adjusted to (6.0 ± 0.1) and the resulting solution was then spiked with the stock solutions of the salicylates in methanol (experiments were performed separately for the two salicylates) to get the concentration of 0.5 mg/L (we are not aware of the existence of reported concentrations for BzS or PS in swimming pools). Similar experiments were performed with the pH of each chlorinated aqueous solution adjusted to (7.0 ± 0.1) and (8.0 ± 0.1). Reactions were studied at room temperature: (20.0 ± 1.0)°C. These experimental conditions were chosen with the intent to include the conditions used in swimming pool and hot tubs. The solutions were kept in the dark and were stirred during the whole experiment. For each experiment, after a reaction time of 20 min, an aliquot of the reaction mixture was taken, the excess of chlorine was quenched with ascorbic acid, and the sample was immediately analyzed by HPLC-UV-DAD. A blank solution with only the salicylate (without chlorine) was also analyzed.

2.3. Study of the reaction kinetics of BzS and PS in chlorinated water

For this study stock solutions of salicylates in methanol (about 100 mg/L) were first prepared. The kinetics of the reactions was evaluated at room temperature: (20.0 ± 1.0)°C. The reactions were carried out in glass vessels containing 100 mL of deionized water. The deionized water samples were spiked with free chlorine to get the initial concentration of 5 mg/L. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl and the resulting solution was then spiked with the stock solutions of the salicylates in methanol (experiments were performed separately for the two salicylates) to get the concentration of 0.5 mg/L. These experimental conditions were chosen with the intent to mimic swimming pool conditions and allow the development of pseudo-first order conditions (Deborde and von Gunten, 2008). This solution was kept in the dark and was stirred during the whole experiment. At fixed reaction times (from 20 to 360 min, every 20 min) an aliquot of the reaction mixture was taken, the excess of chlorine was quenched with ascorbic acid, and the samples were then immediately analyzed by HPLC-UV-DAD. A blank solution with only the salicylate (without chlorine) was also analyzed.

2.4. Identification of DBPs

The identification of the generated DBPs was performed using reaction conditions similar to those used in the kinetics study but the water samples were spiked with the salicylates and chlorine to get higher initial concentrations: 1 mg/L and 10 mg/L, respectively. After (40 min for BzS and 20 min for PS) the water samples (100 mL) were concentrated by liquid–liquid extraction with ethyl acetate (three extractions with 20 mL each). After this procedure the organic solvent was evaporated and 1 mL of methanol was added to the final residue for HPLC-MS analysis. The solutions were kept in the dark till they were analyzed. Yields of the liquid–liquid extraction ($n = 4$ replicates) were calculated by external calibration. Under the conditions used recoveries over 80% were achieved for 100 mL of solution. Although the performance of the analytical procedure for potential by-products of the two salicylates was not evaluated, it was assumed that they behave in a similar way as the parent compounds in regards to the liquid–liquid extraction procedure.

2.5. *Chromatographic conditions*

The reaction kinetics of the salicylates with chlorine was studied by HPLC-UV-DAD. The chromatographic system was constituted by a isocratic pump (Hewlett–Packard 1100 Series, Boeblingen, Germany), a manual sample injection valve with a 20 μL loop (Rheodyne 7725i, Rohnert Park, USA), a silica-based C18 reversed phase column (Hypersil GOLD Column 150 mm × 2.1 mm, particle size 5.0 μm, pore diameter 175 Å, Thermo Scientific, USA) and a photodiode array detector (UV 6000LP with a 50 mm LighPipe flow cell, Thermo Scientific, San Jose, USA). The mobile phase was composed by acetonitrile and water (80%:20%, v/v) for BzS and methanol and water (80%:20%, v/v) for PS. Elutions were performed at a constant flow rate (0.5 mL/min for BzS and 0.25 mL/min for PS) under isocratic conditions. Absorbance was monitored at a total scan mode from 210 to 600 nm. The system was controlled by Xcalibur version 1.4 SR.

The response of the HPLC-UV-DAD system was evaluated according to the ICH guidelines (ICH, 2005) for both salicylates. The linearity of the system was determined from seven standard concentrations between 0.2 and 10 mg/L. Correlation coefficients (R) of the resulting graphs were always higher than 0.999 and the quantification limits remained under 0.1 mg/L.

Chlorination by-products were identified by using a HPLC-MS system composed of a HPLC pump (Finnigan Surveyor LC Pump Plus), an autosampler (Finnigan Surveyor Autosampler Plus) and a photodiode array detector equipped with a LightPipe flow cell (Finnigan Surveyor PDA Plus Detector) (all instrumentation from Thermo Electron Corporation, Waltham, USA), together with a silica-based C18 reversed-phase column (Hypersil GOLD Column 150 mm × 2.1 mm, particle size 5.0 μm, pore diameter 175 Å, Thermo Scientific, USA). In each run a sample of 10 μL was injected. The mobile phases and elution rates (isocratic conditions) used for BzS and PS were the same as the ones used in the reaction kinetics study. Absorbance was monitored at a total scan mode from 220 to 750 nm. The mass spectrometer was a Finnigan LCQ Deca XP Max (Thermo Electron Corporation, Waltham, USA) coupled to the HPLC system. This device was equipped with an electrospray interface as ionization source and a quadrupole ion trap for mass spectrometry experiments, and was operated in the negative ion mode between *m/z* 160 and 600 with the following conditions: spray voltage, 5.0 kV; capillary voltage, 15 V; capillary temperature, 300 °C. The system was controlled by Xcalibur version 1.4 SR.

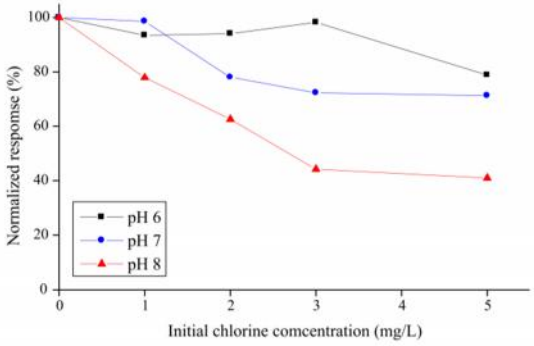

Fig. 2 – Influence of pH and free chlorine concentration on the stability of BzS. Concentrations are related to the corresponding control. Reaction time 20 min.

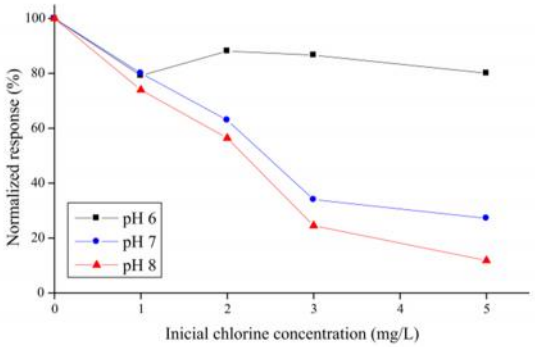

Fig. 3 – Influence of pH and free chlorine concentration on the stability of PS. Concentrations are related to the corresponding control. Reaction time 20 min.

3. Results and discussion

3.1. *Study of the effect of sample pH and free chlorine concentration*

The relevance of reactions between BzS and PS and free chlorine depends on parameters such as the concentration of chlorine and the pH of the water. The variation of the concentration of both salicylates in the presence of different concentrations of free chlorine (0, 1, 2, 3 and 5 mg/L) and for three pH values (6.0, 7.0 and 8.0) is shown in Figs. 2 and 3, respectively for BzS and PS. Normalized responses represented in the Y-axis of both figures correspond to the ratio between the peak area of the salicylate after 20 min reaction and that measured at zero time; therefore, they serve as a rough estimation for the yield of depicted transformations. From Figs. 2 and 3 we can conclude that at pH 6.0 there is a small transformation of both salicylates (maximum about 20%) and is almost independently from free chlorine concentration. At pH 7.0 and 8.0 and for free chlorine concentrations from 2 to 5 mg/L the transformation percentage of BzS

Table 1 – HPLC-MS results from the analysis of DBPs of BzS.

Retention time (min)	Molecular ion (*m/z*)	Proposed formula	Abbreviation[a]
3.37	227.47	$C_{14}H_{12}O_3$	BzS
4.45	261.47	$C_{14}H_{11}O_3Cl$	Cl-BzS (1)
5.17	261.47	$C_{14}H_{11}O_3Cl$	Cl-BzS (2)
7.60	295.33	$C_{14}H_{10}O_3Cl_2$	Cl_2-BzS

[a] Cl-BzS (1) and Cl-BzS (2) refer to isomeric monochlorinated by-products; Cl_2-BzS refers to dichlorinated by-product.

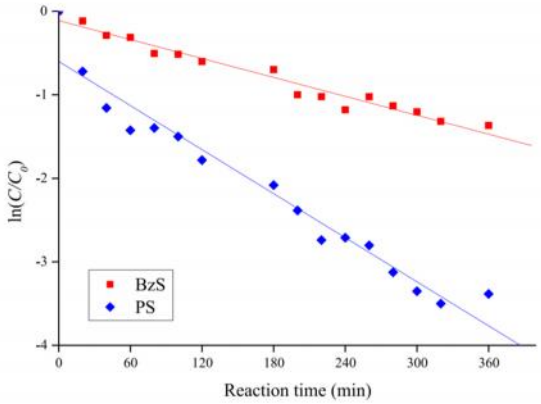

Fig. 4 – ln (C/C_o) *vs* Reaction time for BzS and PS during reaction with chlorine at pH 7.0, room temperature and in the dark. Concentrations are related to the corresponding control.

and PS increases with the increase of the solution pH. This can be due to the fact that the anionic forms of the salicylates (phenoxide anions) react faster with HOCl than the neutral forms with OCl^- ($pK_{HOCl,25°C} = 7.54$, (Deborde and von Gunten, 2008)). We can also conclude that at higher pH values PS reacts with chlorine in a higher extent than BzS.

3.2. *Study of the reaction kinetics of BzS and PS in chlorinated water*

The variation of the concentration of both salicylates with time, in the presence of chlorine, was followed by HPLC-UV-DAD. In Fig. 4 we represent $\ln(C/C_o) = f(t)$ for BzS and PS. Experiments were performed at pH 7.0, room temperature and kept in the dark. Using a ratio of UV filter to chlorine of 0.5:5 the reaction of salicylates with chlorine followed a pseudo-first order kinetics: $\ln(C/C_o) = -0.0038t$; $R^2 = 0.964$ for BsZ and $\ln(C/C_o) = -0.0088t$; $R^2 = 0.954$ for PS. The obtained rate constants were: $k = (0.0038 \pm 0.0002)$ min^{-1} for BzS and $k = (0.0088 \pm 0.0005)$ min^{-1} for PS and the measured half-lives were: $t_{1/2} = (182 \pm 10)$ min for BzS and $t_{1/2} = (79 \pm 4)$ min for PS (mean ± standard deviation). At the end of the experiments the transformation percentages for BzS and PS were found to be 71.4% and 93.0%, respectively. BzS was found to be more stable in the presence of chlorine than PS. This can be due to the fact that the transition state between the parent molecule and the DBP is more stabilized in PS due to the possibility of π electron delocalization between the phenol and the benzene moieties through the ester group and this stabilization is precluded in BzS due to the presence of the methylene moiety.

3.3. *Identification of DBPs*

HPLC and MS results obtained from the mixture resulting from the reaction of BzS with chlorine at pH = 7.0 in the dark are given in Supplementary Material (Fig. S1–S5). Retention times and most intense ions in the MS spectrum of the DBPs of BzS are shown in Table 1. By interpretation of the mass spectra it was possible to tentatively identify three DBPs. Compounds appearing at retention times 4.45 and 5.17 exhibited one peak with $m/z = 261$ and probably correspond to two mono-chlorinated BzS isomers with molecular formula $C_{14}H_{11}O_3Cl$. The peak with $m/z = 295$ corresponds to a dichloro- by-product with molecular formula $C_{14}H_{10}O_3Cl_2$. The mono- and dichloro- BzS compounds are most probably the result of replacement of one and two hydrogen atoms per one and two chlorine atoms in the phenolic ring. Positions were those replacements occur could not be determined experimentally. According to Deborde and von Gunten (2008) chlorine reacts with compounds containing aromatic rings mostly by electrophilic substitutions mainly in *ortho* and *para* positions to a substituent. Considering the structure of BzS (Fig. 1(a)) and the activation effect of the hydroxyl group towards electrophilic

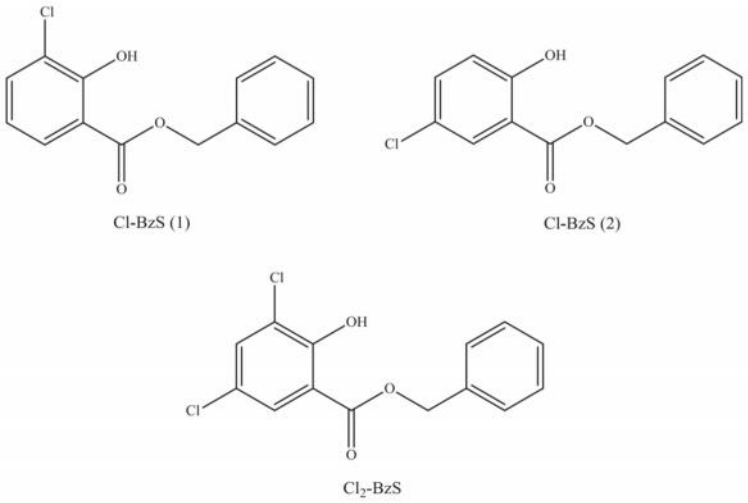

Fig. 5 – Proposed structural formula of the detected DBPs of BzS: monochloro- (Cl-BzS (1) and Cl-BzS (2)) and dichloro- (Cl_2-BzS).

Table 2 – HPLC-MS results from the analysis of DBPs of PS.

Retention time (min)	Molecular ion (*m/z*)	Proposed formula	Abbreviation[a]
3.93	213.36	$C_{13}H_{10}O_3$	PS
4.77	247.42	$C_{13}H_9O_3Cl$	Cl-PS (1)
5.89	247.28	$C_{13}H_9O_3Cl$	Cl-PS (2)
8.56	281.55	$C_{13}H_8O_3Cl_2$	Cl_2-PS

[a] Cl-PS (1) and Cl-PS (2) refer to isomeric monochlorinated by-products; Cl_2-PS refers to dichlorinated by-product.

substitution reactions, the most probable ones are the *ortho* and *para* positions relative to this group. Proposed structures for the mono- and dichlorinated by-products of BzS are presented in Fig. 5.

HPLC and MS results obtained from the mixture resulting from the reaction of PS with chlorine at pH = 7.0 in the dark are given in Supplementary Material (Fig. S6–S10). Retention times and most intense ions in the MS spectrum of the DBPs of PS are shown in Table 2. By interpretation of the mass spectra it was possible to tentatively identify three DBPs. The compounds appearing at retention times 4.77 and 5.89 min exhibited a molecular ion at $m/z = 247$ which corresponds to a monochloro-by-product with molecular formula $C_{13}H_9O_3Cl$. The compound appearing at retention time 8.56 min exhibited a molecular ion at $m/z = 281$ which corresponds to a dichloro-by-product with molecular formula $C_{13}H_8O_3Cl_2$. Similarly to BzS substitution of hydrogen atoms by chlorine occurs most probably on the phenolic ring in the *orto* and *para* positions relative to the hydroxyl group (see Fig. 1(b) and Fig. 5).

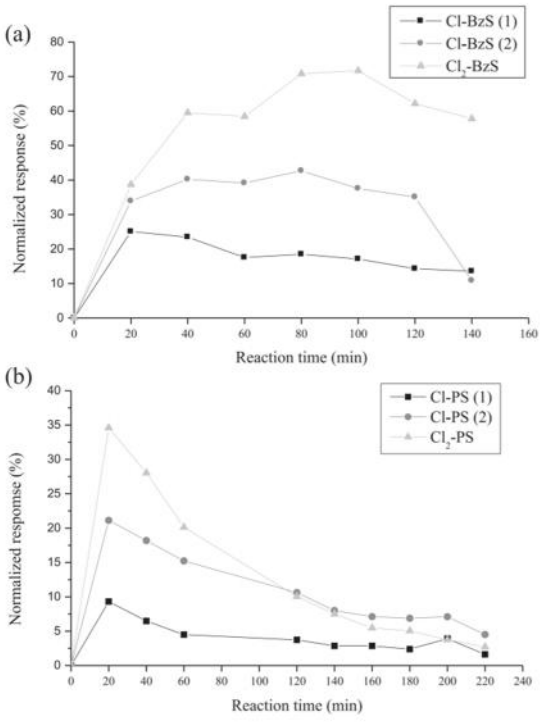

Fig. 6 – Time course of some DBPs (pH = 7.0; 10 mg/L of chlorine; 1 mg/L salicylate, dark) for (a) BzS and (b) PS.

3.4. *Stability of the DBPs*

The stability of the mono- and dichlorinated by-products of both salicylates was followed from 20 to 140 min, every 20 min for BzS and from 20 to 220 min, every 20 min for PS, considering a concentration of 1 mg/L of salicylate, 10 mg/L of free chlorine and sample pH of 7.0. Results are plotted in Fig. 6(a) for BzS and Fig. 6(b) for PS. Normalized responses represented in the Y-axis correspond to the ratio between the peak area of each DBP, at a given, reaction time, and that measured for the parent salicylate in the reference experiment at zero time; therefore, they serve as a rough estimation for the yield of depicted transformations. BzS by-products showed a good stability under the experimental conditions used (Fig. 6(a)) and in the measurement period. After about 2 h the DBPs are still present. In the case of PS by-products all DBPs reached a maximum after 20 min and then decreased to around 5% in about 3 h.

4. Conclusions

The studied salicylates BzS and PS react with chlorine leading to the formation of DBPs. PS was found to be less stable in chlorine and we think this reactivity difference is due to the possibility of a higher stabilization of the transition state of the electrophilic substitution reaction. DBPs of the two salicylates have been tentatively identified by HPLC-MS. Two mono- and one dichlorinated by-product have been detected for both salicylates. In contrast, DBPs of BzS showed a good stability while PS by-products were found to significantly degrade after 20 min reaction.

From the point of view of the water quality of swimming pools where the PCPs used by swimmers are released to water this work is quite relevant because these PCPs and their transformation products accumulate in the bathing water rising human health concerns. Environmental risk can also arise after discharge of the swimming pool effluents into the aquatic systems.

Future work should investigate the presence of these DBPs in swimming pool water and waste water, address potential risks for human health due to dermal contact and evaluate possible environmental toxic effects. Environmental chemistry studies should also focus on strategies to minimize the formation of these DBPs by the development of new formulations that prevent PCPs release into chlorinated water and strategies to reduce PCPs by pool water treatment and by pre-swim showering.

Acknowledgment

M. S. Miranda thanks Fundação para a Ciência e a Tecnologia, FCT, Lisbon, Portugal, for the financial support under the frame of the Ciência 2008 program.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.07.018.

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Study of the transformation of two salicylates used in personal care products in chlorinated water

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Supplementary information

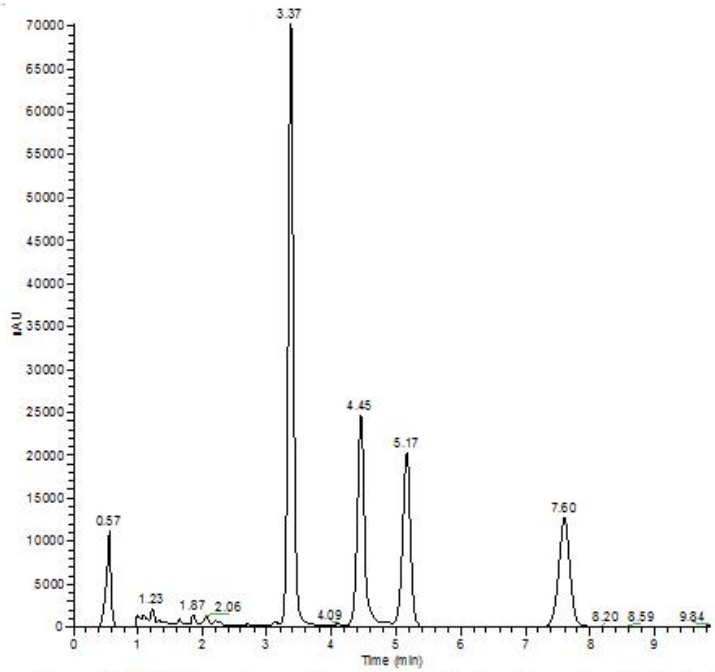

Figure S1 – HPLC chromatogram of the mixture resulting from the reaction of BzS with chlorine.

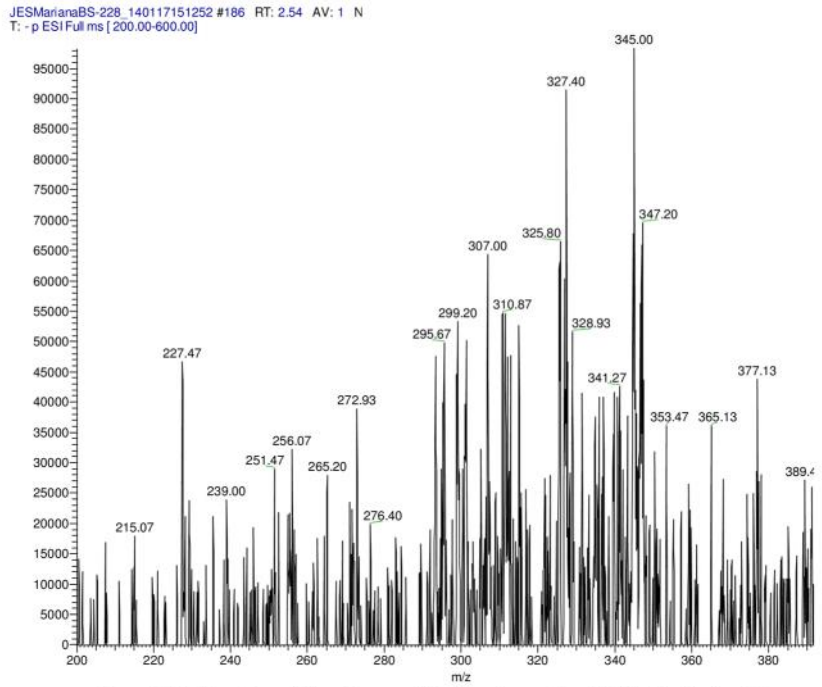

Figure S2 - MS spectrum of the mixture resulting from the reaction of BzS with chlorine: BzS.

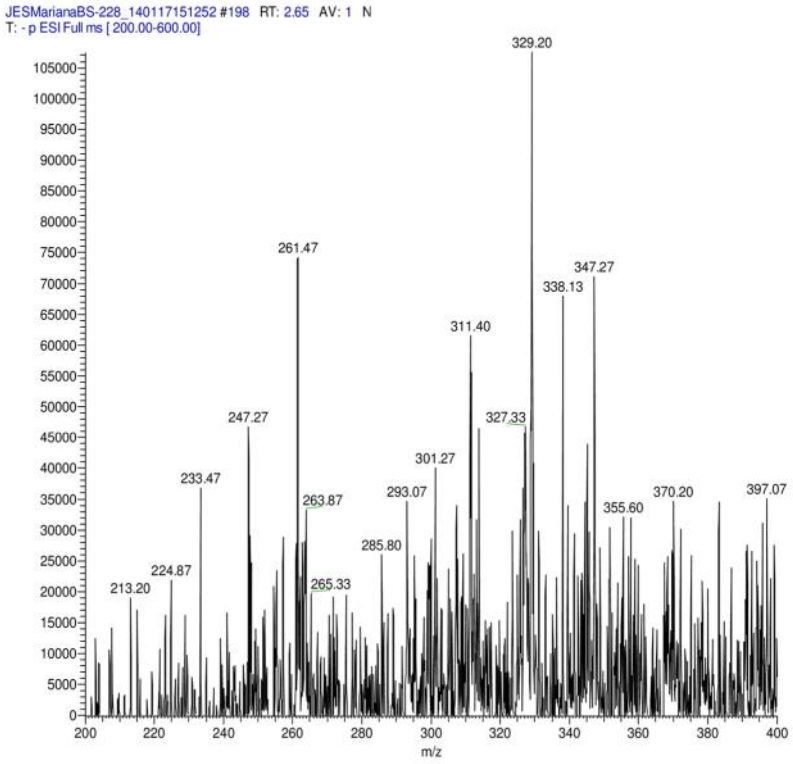

Figure S3 - MS spectrum of the mixture resulting from the reaction of BzS with chlorine: Cl-BzS (1).

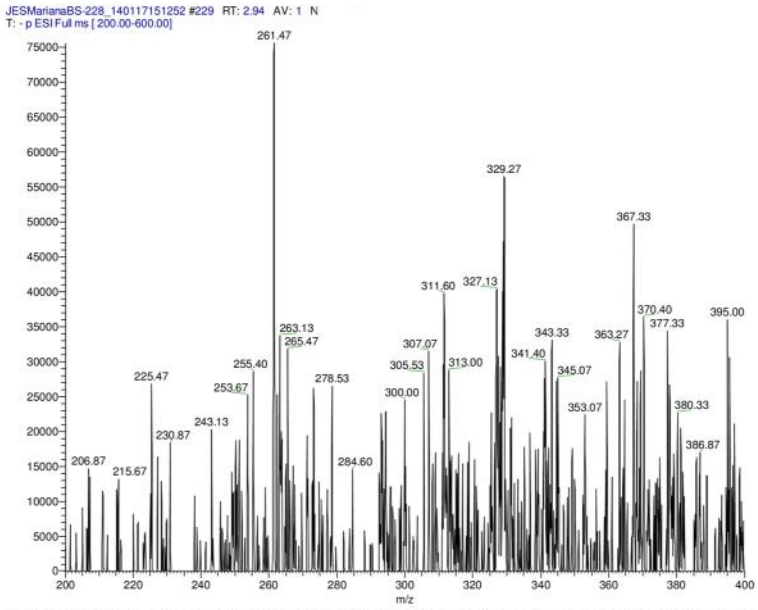

Figure S4 - MS spectrum of the mixture resulting from the reaction of BzS with chlorine: Cl-BzS (2).

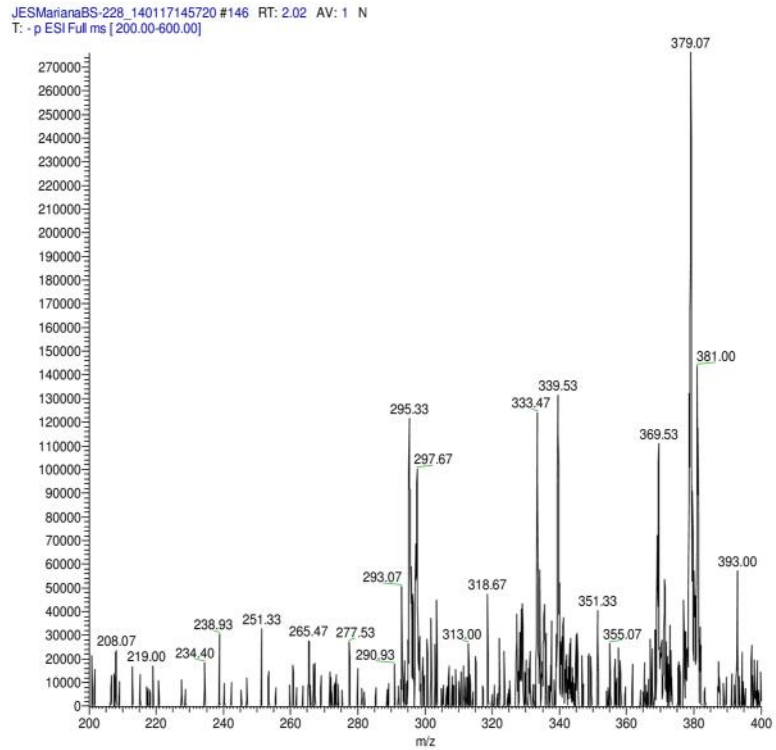

Figure S5 - MS spectrum of the mixture resulting from the reaction of BzS with chlorine: Cl_2-BzS.

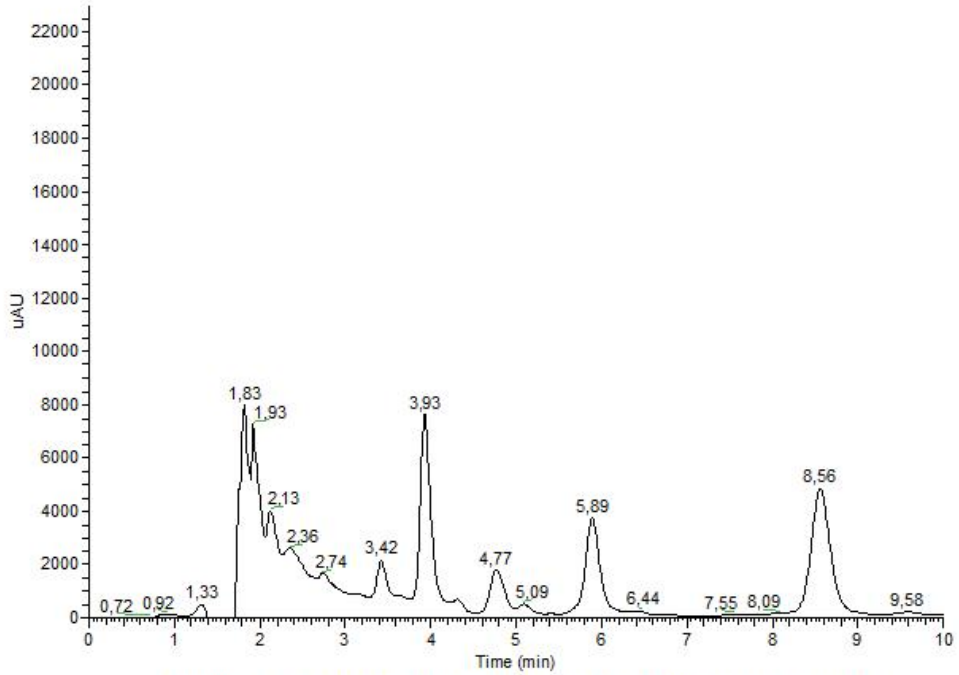

Figure S6 - HPLC chromatogram of the mixture resulting from the reaction of PS with chlorine.

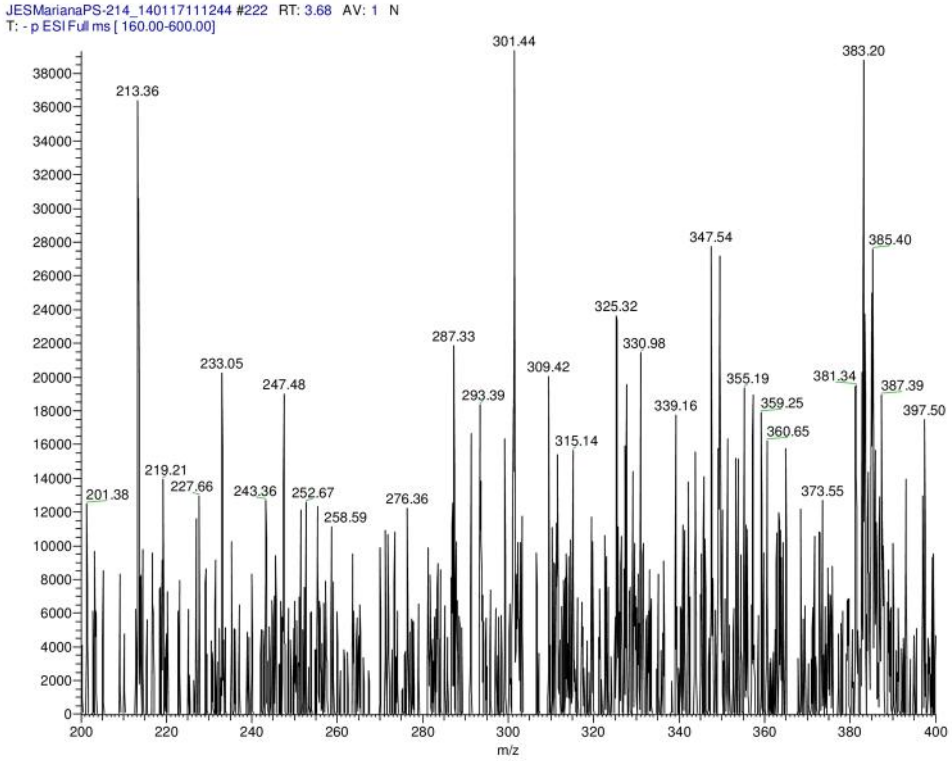

Figure S7 - MS spectrum of the mixture resulting from the reaction of PS with chlorine: PS

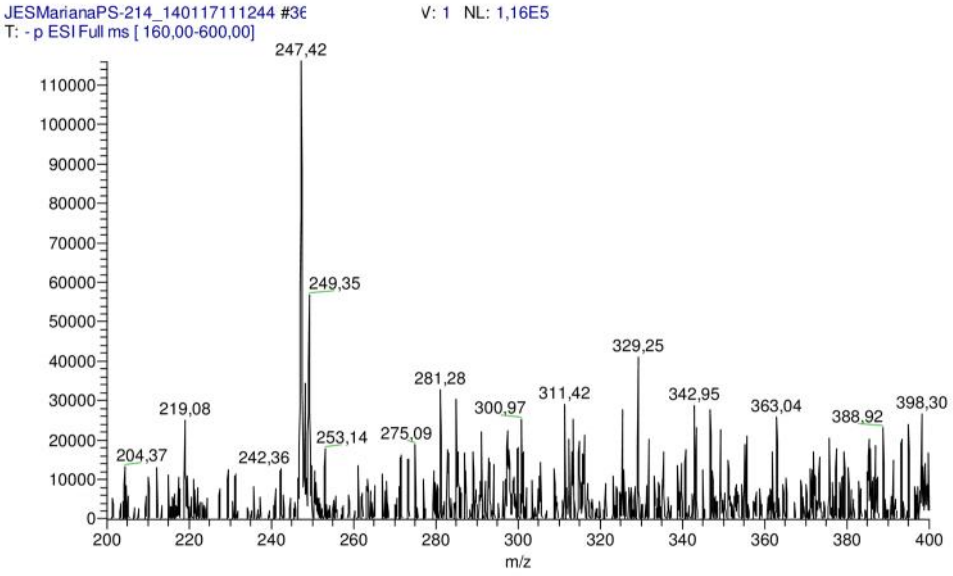

Figure S8 - MS spectrum of the mixture resulting from the reaction of PS with chlorine: Cl-PS (1).

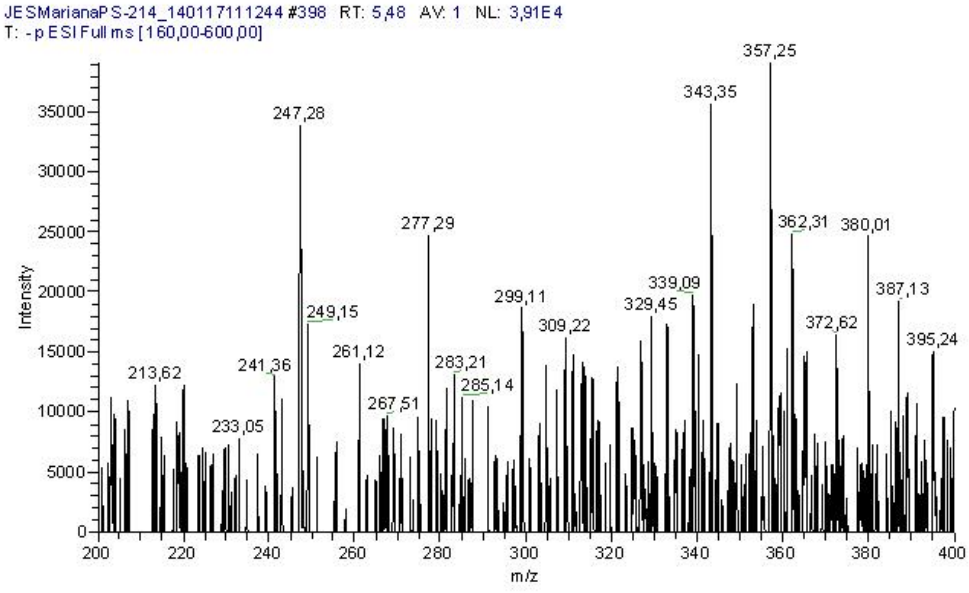

Figure S9 - MS spectrum of the mixture resulting from the reaction of PS with chlorine: Cl-PS (2).

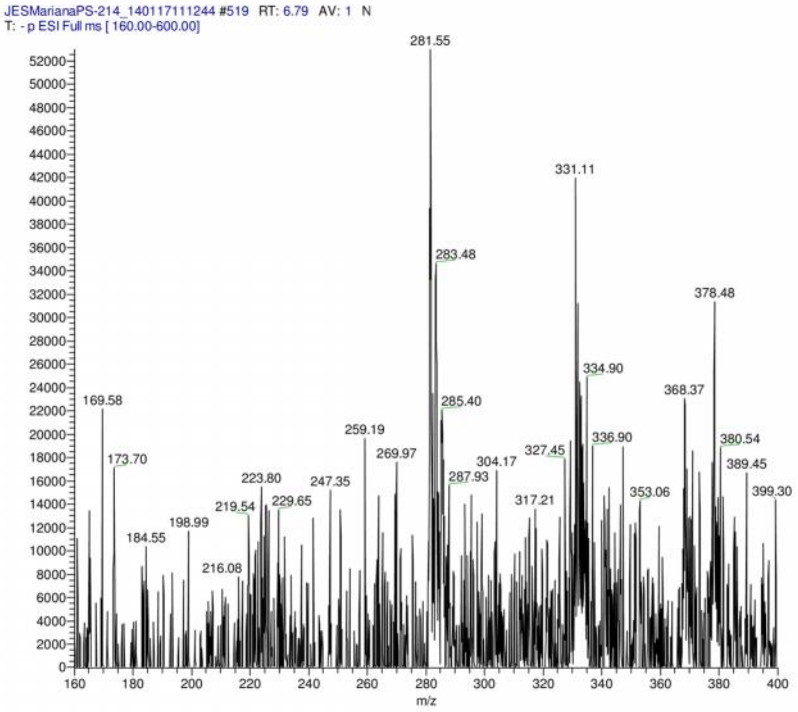

Figure S10 - MS spectrum of the mixture resulting from the reaction of PS with chlorine: Cl_2-PS.

Chapter IV

Study of the transformation of 4-MBC and OC in chlorinated water and by UV radiation

4. Study of the transformation of 4-MBC and OC in chlorinated water and by UV radiation

4.1. Photodegradation of UV-filters

Besides the reaction between chlorine and UV-filters described above (Chapter II), these compounds may also suffer transformation induced by sunlight.

These photochemical reactions are one of the most important abiotic processes determining the aquatic fate of organic compounds in natural waters (Rodil *et al*., 2009). It is important that UV-filters are stable under sunlight exposure because a high screening efficiency is only guaranteed if the UV-filter has high stability (Santos *et al.*, 2012; Rodil *et al*., 2009). In a commercial formula of sunscreen, these photostability depends not only on the UV-filter but also of the presence of other UV-filters, other active substances, like antioxidants (Gaspar and Maia Campos, 2007). However, several papers have been published reporting photodegradation of some UV-filters. In 2006, Gaspar and Campos (Gaspar and Maia Campos, 2006) analyzed the photostability of four different UV-filters combinations after UVA and UVB irradiations: EHMC, BP3 and octyl salicylate (OS) (formulation 1); EHMC, BDM and 4-MBC (formulation 2); EHMC, BP3 and OC (formulation 3); and EHMC, BDM and OC (formulation 4). They studied UV-filters formulations instead individual UV-filters because the behavior of sunscreens is not predictable from the photostability of its individual filter but from the behavior of their UV-filters combinations (Gaspar and Maia Campos, 2006). So, they observed that, in terms of EHMC recovery, formulation 3 was the most stable, followed by formulation 4, formulation 1 and formulation 2. In terms of BDM recovery formulation 4 was more stable than formulation 2, and regarding BP3 recovery formulation 3 was more stable than formulation 1. In terms of OC recovery, formulation 3 was more stable than formulation 4. It was observed that BDM and EHMC react with each other during irradiation time forming cycloaddition products and maybe other photoadducts, fact that may explain why formulation 3 was more stable than formulations 4 and 2. In this study, it was also observed that OC and 4-MBC can stabilize BDM, OC is very effective in stabilizing EHMC in presence of BDM and OC is good UV stabilizer, since formulation 3, which contained EHMC, BP3 and OC, was more stable than formulation 1, which contained EHMC, BP3 and OS.

Concerning BDM, Huong *et al*. (Huong *et al*., 2008) observed that, under irradiation in aqueous solution, the *enol* form tautomerizes to the *keto* form and is also fully degraded. Substituted benzoic acids, benzils, dibenzoylmethanes and dibenzoyl ethanes were the photoproducts found by Huong *et al*. (Huong *et al*., 2008).

In 2003, Sakkas *et al*. (Sakkas *et al*., 2003) studied photodegradation of another UV-filter: EHDPABA. In this study, differently to the paper referred above, photodegradation was analyzed in different types of water: distilled, chlorinated pool and seawater. It was observed that reaction rates followed the order: distilled water>swimming pool water>seawater. After 60h of natural irradiation (daylight exposure) 86, 83 and 80% of this UV-filters as degraded in distilled, swimming pool and seawater, respectively. However, simulated solar irradiation was more effective in EHDPABA: only 10h were enough to degrade more than 99, 96 and 92%, respectively. It was also observed that the presence of organic matter like humic acids inhibits the photodegradation rate of EHDPABA. This retardation on the degradation rate occurs maybe due to a competition process between organic matter and the UV-filter for the available photons. Incident light scattered by particulate matter suspended in the water column and a partial binding between organic matter and EHDPABA by hydrophobic partitioning or weak van der Waals forces which causes a fraction that will never be available to photolysis action are another reasons to the retardation on photodegradation rate. In seawater, this is also consistent with •OH scavenging by chloride ions.

Rodil *et al.* (Rodil *et al*., 2009) analyzed the stability of six UV-filters exposed to artificial sunlight in water: BP3, ethylexyl methoxycinnamete (EHMC), isoamyl methoxicinnamte (IAMC), EHDPABA, OC and 4-MBC. Half-lives and phototransformation products of these UV-filters were identified. And they observed that BP3, OC and 4-MBC are very photostable, while EHMC, IAMC and EHDPABA clearly showed photodegradation. It was also observed a fast *E* to *Z* isomerization of EHMC, IAMC and 4-MBC. This isomerization phenomenon was confirmed as a way to reduce the absorber UV energy. Isomerization of EHMC was previously observed by Huong (Huong *et al.*, 2007).

Concerning photodegradation products, Rodil *et al.* (Rodil *et al*., 2009) found two products resulting from dealkylation of EHDPABA, a photodissociation process whereby the loss of one and two of the methyl groups of EHDPABA occurs, respectively. These photoproducts showed to be stable for several days. Another compound was also observed and it was proposed as a methylated derivate of the parent compound. These photoproducts showed to be stable for several days. On the other hand, no photodegradation product was detected for EHMC and IAMC.

Few years later Liu *et al*. (Liu *et al.*, 2011) reported again the significant photostability of BP3 under both artificial and natural sunlight. However, in this study BP3 was significantly degraded when in contact with another UV-filter (benzotriazole) and with humic acids in solution. In this case, it was detected one photoproduct produced through the loss of hydroxyl and benzoyl functional group. This product was determined and identifies as 2,4-dimethylanisole.

The photostability of *p*-aminobenzoic acid (PABA), EHDPABA, EHMC, BP3, PBSA in different solvents was studied by Serpone *et al.* (Serpone *et al.*, 2002): water, methanol, acetonitrile and n-hexane, under aerobic and anaerobic conditions. It was observed that in both types of solvents (aqueous and non-aqueous) PABA had an extensive photodegradation: 87%, 65%, 60% and 45% in n-hexane, water, methanol and acetonitrile respectively. On the other hand EHDPABA had a higher degradation in n-hexane (97%) followed by acetonitrile (94%), water (75%) and methanol (15%). Regarding photodegradation of EHMC, 90% of this UV-filter was degraded in water, 45% in acetonitrile and 40% in methanol and in n-hexane. These percentages were obtained after 30 min of UV exposure, however after 2h of UV exposure EHMC degraded in 95% in n-hexane yielding several photodegradation products. Similar to Rodil *et al.* (Rodil *et al*., 2009) and to Liu *et al*. (Liu *et al.*, 2011), in this study Serpone *et al.* (Serpone *et al.*, 2002) also observed a high stability of BP3 in water (20% of degradation after 2h of UV exposure) as well as in acetonitrile and n-hexane (5-10% and 15% of degradation, respectively, after 2h of UV exposure). However, in methanol BP3 was particularly unstable, photodegrading almost completely (90%) after 2 h. Finally, PBSA showed a high insolubility in n-hexane but its degradation was very fast in water (90% after only 10 min of UV exposure). In acetonitrile the degradation was nearly by 50% after only 20 min of UV exposure and 70% complete after 2 h. The degradation was also significant in methanol.

Serpone *et al.* (Serpone *et al.*, 2002) also observed that PABA, EHDPABA, EHMC and BP3 have a faster degradation under aerobic than under anaerobic conditions: 60% vs. 55% after 1h for PABA, 55% vs. 20% after 10 min for EHDPABA, 85% vs. 65% after 20 min for EHMC, and 50% vs. 15% after 260 min for BP3, respectively. This happens because oxygen is converted in some reactive oxygen species. In the case of PABA, this UV-filter is a good photosensitizer when exposed to UV radiation yielding molecular oxygen. PABA is also a good sink for these reactive oxygen species which causes PABA self-destruction.

In 2010, Zhang *et al.* (Zhang *et al*., 2010) confirmed that PBSA photodegradates under artificial UV radiation in aqueous solution and they identified several photodegration products in pure water: a desulfonated product (2-phenylbenzoimidazole), three products formed from the cleavage of the benzene ring adjacent to the imidazole ring (phenylimidazolecarboxylic acid derivatives) and two compounds produced from the opening of the imidazole ring (benzimidamide and benzamide).

The aim of this step was: (i) assess the stability of 4-MBC and OC in chlorinated water, in conditions similar to those existent in swimming pools, and (ii) assess the stability of 4-MBC and OC under artificial and natural UV radiation.

4.2. UV-filters under study

4-MBC is an UV-filter used in many PCPs like sunscreens, lotions and shampoos being one of the most commonly used UV-filters (Buser *et al*., 2005). This UV-filter is one of the authorized UV filters in Europe and Australia and has been submitted for approval in the USA (Scalia *et al.*, 2007). It absorbs in UVB region (290-320 nm) (Giokas *et al.*, 2007; Scalia *et al.*, 2007) and can exist as a *cis*- (*Z*) and *trans*- (*E*) isomer due to exocyclic carbon-carbon double bound (Fig.4.1). Although only the *E*-form is present in commercial products, both isomers can be found in environmental samples (Buser *et al*., 2005). 4-MBC has a good photostabilizer effect in the sunscreens formulations.

OC is another UV-filter introduced in sunscreens products approximately 14 years ago (Avenel-Audran *et al.*, 2014). This UV-filter is an ester (Fig.4.2) formed by the condensation of a diphenylcyanoacrylate with 2-ethylhexane (Avenel-Audran *et al.*, 2014) and it absorbs in UVB region (390-360 nm) (González *et al*., 2008). OC has been increasingly used due to its spectrum efficiency covering UVB but also short UVA wavelengths (Avenel-Audran *et al.*, 2014). It is photostable (González *et al*., 2008) and it is also used as photostabilizer in sunscreens formulations (Palm and O'Donoghue, 2007).

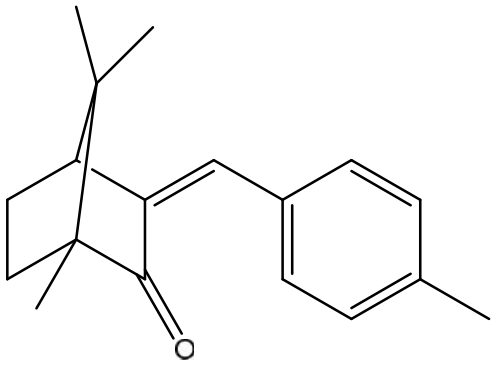

Figure 4.1. Structure formula of 4-MBC.

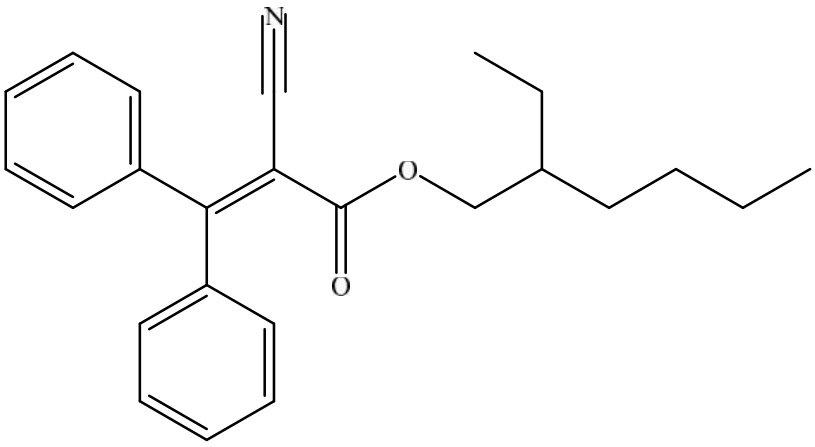

Figure 4.2. Structure formula of OC.

4.3. Materials and Methods

4.3.1. Reagents

4-MBC, CAS Registry No. [36861-47-9], 98%, (molecular formula $C_{18}H_{22}O$) was purchased from Sigma-Aldrich. OC, CAS Registry No. [6197-30-4], 98%, (molecular formula $C_{24}H_{27}O_2$) was purchased from Aako. A commercial sodium hypochlorite solution with a chlorine content of <5% (28 mg/L of free chlorine) was used in the chlorination studies. This solution was stored at 4ºC and its free chlorine content was periodically measured by a small compact photometer for chlorine (HANNA Checker Mini Hi 701 series). Stock solutions of the UV-filters were prepared with methanol from Merck. Deionized water (conductivity < 0.1 $\mu S\ cm^{-1}$) was used in all experiments. Ascorbic acid (99.7%) used to stop the chlorination reaction was obtained from Merck. The pH of the solutions was adjusted to a pre-determined value with HCl solutions 0.1 mol/L. Eluents for chromatographic analysis (methanol and acetonitrile) were liquid chromatographic grade and were bought from Merck.

4.3.2. Study of the reaction kinetics of 4-MBC and OC in chlorinated water

For this study stock solutions of the two UV-filters in methanol (about 100 mg/L) were first prepared. The kinetics of the reactions was evaluated at room temperature: (20.0 ± 1.0)ºC. The reactions were carried out in glass vessels containing 100 mL of deionized water. At first, the deionized water samples were spiked with free chlorine to get the initial concentration of 10 mg/L for the two UV-filters. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl and the resulting solution was then spiked with the stock solutions of the UV-filters in methanol (experiments were performed separately for each one) to get the concentration of 1 mg/L (ratio UV-filter:chlorine 1:10). These experimental conditions were chosen with the intent to mimic swimming pool conditions and allow the development of pseudo-first order conditions (Deborde and Von Gunten, 2008). These solutions were kept in the dark and were stirred during the whole experiment. At fixed reaction times (every 20 min for 4-MBC; and from 20 to 4162 min for OC) an aliquot of the reaction mixture was taken, the excess of chlorine was quenched with ascorbic acid according the stoichiometric relationship of 2.5 parts of ascorbic acid to 1 part of free chlorine, and the samples were then immediately analyzed by HPLC-UV-DAD. A blank solution with only the UV-filter (without chlorine) was also analyzed.

The ratios UV-filter:chlorine 1:20 and 0.25:1 were also studied for OC.

4.3.3. Study of the degradation induced by UV radiation of 4-MBC and OC

In a first stage, to study the photodegradation of 4-MBC, a 30 min assay analyzed by a spectrophotometer Hewlett Packard 8452A Diode Array and also by HPLC-UV-DAD was conducted. In this step stock solutions of 4-MBC in methanol (about 100 mg/L) were first prepared. The reactions were carried out under artificial sunlight UV radiation in glass vessels containing 100 mL of deionized water spiked with the stock solutions of the UV-filter to get the concentration of 1 mg/L. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl. This solution was kept during the whole experiment under artificial UV radiation provided by a mercury lamp (PHILIPS, HPLR 400W). The distance between the water surface and the lamp was 5.5 cm. The temperature of the solutions increased during the experiment due to the heat released by the lamp (from 23.8 ºC to 26.5 ºC). At fixed reaction times (from 10 to 30 min) an aliquot sample of the reaction mixture was taken and the samples were then immediately analyzed by spectrophotometer and HPLC-UV-DAD. It was also analyzed a blank solution with the UV-filter kept in the dark but suffering the same temperature fluctuation.

This 30 min assay was performed also under natural UV radiation. In this case, the reactions were carried out separately in glass vessels containing 100 mL of deionized water spiked with the stock solutions of 4-MBC in methanol to get the concentration of 1 mg/L. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl. Blank solutions with the UV-filter kept in the dark was also analyzed. At fixed reaction times (from 10 to 30 min, every 10 min) an aliquot of the reaction mixture was taken and the samples were then immediately analyzed by spectrophotometer and HPLC-UV-DAD. To study the behavior of OC under UV radiation, stock solutions of OC in methanol (about 100 mg/L) were first prepared. The reaction was carried out under artificial UV radiation in glass vessels containing 100 mL of deionized water spiked with the stock solutions of OC to get the concentration of 0.5 mg/L. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl. This solution was kept during 30 min under artificial UV radiation provided by a mercury lamp with (PHILIPS, HPLR 400W). The distance between de water surfaces into the glass vessel and the lamp was 5.5 cm. The temperature of the solutions fluctuated according the increasing of the temperature caused by the heat of the lamp: (24.0 ± 2.0)ºC. After 30 min of reaction an aliquot sample of the reaction mixture was taken and was immediately analyzed by spectrophotometer and HPLC-UV-DAD.

In a second step, stock solutions of 4-MBC and OC in methanol (about 100 mg/L) were first prepared. The degradation was evaluated at constant temperature (20.0 ± 2.0)ºC ensured by a glass vessel with double walls with water circulating at room

temperature. The reactions were carried out in 100 mL of deionized water. The deionized water samples were spiked with the stock solutions of the UV-filters in methanol (experiments were performed separately for each one) to get the concentration of 1 mg/L. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl. These solutions were kept under artificial UV radiation provided by a mercury lamp (PHILIPS, HPLR 400W) emitting in the range of UVB (280 to 315 nm) and were stirred during the whole experiment. The distance between de water surfaces into the glass vessel and the lamp was 5.5 cm. At fixed reaction times (from 30 to 150 min, every 30 min for 4-MBC, and from 60 to 240, every 60 min for OC) an aliquot of the reaction mixture was taken and the samples were then immediately analyzed by HPLC-UV-DAD. A blank solution with the UV-filter kept in the dark was also analyzed.

In addition to artificial source of UV radiation, studies under natural UV radiation were also conducted. For these studies stock solutions of 4-MBC and OC in methanol (about 100 mg/L) were first prepared. The reactions were carried out in glass vessels containing 100 mL of deionized water spiked with the stock solutions of the UV-filters in methanol (experiments were performed separately for each one) to get the concentration of 1 mg/L. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl. These solutions were kept during the whole experiment under direct natural UV radiation. In this case, the temperature fluctuated according to the environmental temperature (between 21 and 28 ºC for 4-MBC and between 24 and 31 ºC for OC). At fixed reaction times (from 60 to 360 min, every 60 min for 4-MBC, and from 60 to 480, every 60 min for OC) an aliquot of the reaction mixture was taken and the samples were then immediately analyzed by HPLC-UV-DAD. It was also analyzed a blank solution with the UV-filter kept in the dark but suffering the same temperature fluctuation.

There was also tested the effect caused by chlorine in these two steps. In the 30 min assay, chlorine was added only to 4-MBC. The reactions were carried out in glass vessel containing 100 mL of deionized water. The deionized water samples were spiked with free chlorine to get the initial concentration of 10 mg/L. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl and the resulting solution was then spiked with the stock solutions of 4-MBC in methanol to get the concentration of 1 mg/L (ratio UV-filter:chlorine 1:10). The solutions were kept under artificial UV radiation provided by a mercury lamp (PHILIPS, HPLR 400W) emitting in the range of UVB (280 to 315 nm) during the whole experiment and the temperature of the solutions fluctuated according the increasing of the temperature caused by the heat of the lamp: (24.0 ± 2.0)ºC. The distance between de water surfaces into the glass vessel and the lamp was 5.5 cm. At each 10 min in a reaction time of 30 min an aliquot of the 4-MBC reaction was taken, the excess of chlorine was quenched with ascorbic acid according the

stoichiometric relationship of 2.5 parts of ascorbic acid to 1 part of free chlorine, and the samples were then immediately analyzed by spectrophotometer and HPLC-UV-DAD. A blank solution kept in the dark with 4-MBC and chlorine and suffering the same temperature fluctuation was also analyzed.

In the second step (at the study along a higher reaction time), it was added chlorine to the 4-MBC and OC reactions. Together with the photodegaration reactions already described, there was also studied the degradation induced by both UV radiation and chlorine. The reactions were carried out in glass vessels containing 100 mL of deionized water. The deionized water samples were spiked with free chlorine to get the initial concentration of 10 mg/L. After that, the pH of the aqueous solution was adjusted to (7.0 ± 0.1) with 0.1 mol/L HCl and the resulting solution was then spiked with the stock solutions of 4-MBC and OC in methanol to get the concentration of 1 mg/L (ratio UV-filter:chlorine 1:10). These solutions were kept under direct natural radiation and their temperature fluctuated according to the environmental temperature (between 21 and 26ºC for 4-MBC and between 24 and 31ºC for OC) during the whole experiment, and also under indirect natural UV radiation provided by a mercury lamp (PHILIPS, HPLR 400W) emitting in the range of UV-B (280 to 315 nm) for OC [at constant temperature: (23.0 ± 2.0)ºC; the constant temperature was ensure by a glass vessel with double walls with water circulating at room temperature]. The distance between de water surfaces into the glass vessel and the lamp was 5.5 cm. An aliquot of the reactions mixtures was taken, the excess of chlorine was quenched with ascorbic acid according the stoichiometric relationship of 2.5 parts of ascorbic acid to 1 part of free chlorine, and the samples were then immediately analyzed by spectrophotometer and HPLC-UV-DAD. A blank solution with only the UV-filter was kept in the dark suffering the same temperature fluctuation was also analyzed.

4.3.4. Chromatographic conditions

The reaction kinetics of the four UV-filters with chlorine and the degradation induced by UV radiation were studied by HPLC-UV-DAD. The chromatographic system was constituted by a isocratic pump (Hewlett-Packard 1100 Series, Boeblingen, Germany), a manual sample injection valve with a 20 μL loop (Rheodyne 7725i, Rohnert Park, USA), a silica-based C18 reversed phase column (Hypersil GOLD Column 150 mm × 2.1 mm, particle size 5.0 μm, pore diameter 175 Å, Thermo Scientific, USA) and a photodiode array detector (UV 6000LP with a 50 mm LighPipe flow cell, Thermo Scientific, San Jose, USA). The mobile phase was composed by acetonitrile and water (80%:20%,

v/v) for BzS and 4-MBC and methanol and water (80%:20%, v/v) for PS and OC. Elutions were performed at a constant flow rate (0.5 mL/min for BzS, 0.25 mL/min for PS, and 0.40 mL/min for 4-MBC and OC) under isocratic conditions. Absorbance was monitored at a total scan mode from 210 to 600 nm. The system was controlled by Xcalibur version 1.4 SR.

The response of the HPLC-UV-DAD system was evaluated according to the ICH guidelines (ICH, 2005) for each UV-filter. The linearity of the system was determined from seven standard concentrations between 0.2 to 10 mg/L. Correlation coefficients (R) of the resulting graphs were always higher than 0.999 and the quantification limits remained under 0.1 mg/L.

4.4. Results and discussion

4.4.1. Study of the reaction kinetics of 4-MBC and OC in chlorinated water

The variation of the concentration of 4-MBC and OC with time, in the presence of chlorine, was followed by HPLC-UV-DAD. Experiments were performed at pH 7.0, room temperature and kept in the dark, at first using a ratio of UV-filter to chlorine of 1:10 and then 5:10. 4-MBC was found not react with chlorine as we can see in Fig.4.3: normalized values in the *Y*-axis correspond to the ratios between the responses for each sample from chlorinated and nonchlorinated aliquots, multiplied by 100. As observed, the concentration present in each aliquot sample doesn't follow the decrease associated to the chlorine reaction, as happens with BzS and PS.

Regarding OC, the variation of its concentration with time, in the presence of chlorine, was followed by HPLC-UV-DAD. Experiments were performed at pH 7.0, room temperature and kept in the dark, at first using a ratio of UV-filter to chlorine of 1:10. It was found that OC did not react with chlorine did not occur in useful time (Fig.4.4). At the end of the experiments (121 h) the transformation percentage was found to be 34.3%. Then, the ratio UV-filter:chlorine was changed to 1:20 to improve the chance of the chlorine reacts with OC (116 h). At the end of the experiments the transformation percentage was found to be 94.5%. Then keeping the same proportion, the ratio UV-filter:chlorine was changed to 0.5:10 and the variation of the concentration of OC with time in the presence of chlorine was followed during 70h. However, 70 h is too much time to study the chlorination reaction of UV-filters.

The variation of the concentration of OC with time using a ratio of UV-filter to chlorine of 0.25:10 was also assessed. The reaction was followed during 390 min and it was observed only 20% of degradation.

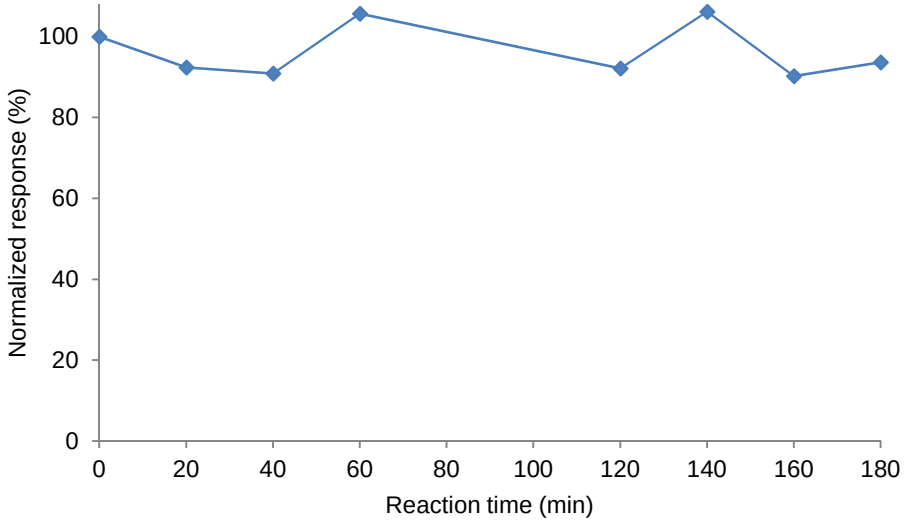

Figure 4.3. C/C_o (%) *vs* Reaction time for 4-MBC during reaction with chlorine at pH 7.0, room temperature and in the dark. Concentrations are related to the corresponding control.

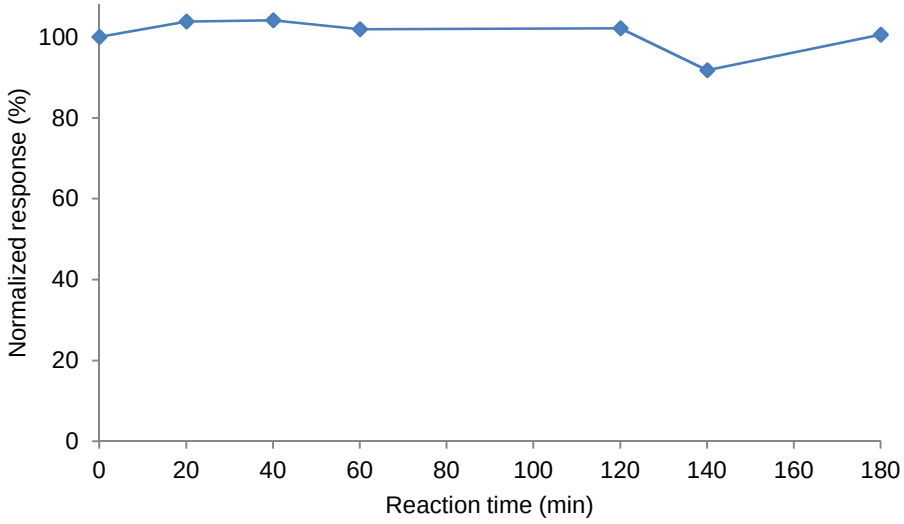

Figure 4.4. C/C_o (%) *vs* Reaction time for OC during reaction with chlorine at pH 7.0, room temperature and in the dark. Concentrations are related to the corresponding control.

4.4.2. Study of the degradation induced by UV radiation of 4-MBC and OC

Once the reaction with chlorine was not observed and to increase the knowledge about the reactions suffered by 4-MBC and OC in swimming pool water, the degradation induced by UV radiation was studied. In a first stage, the photodegradation of 4-MBC and OC was evaluated during 30 min.

Two solutions with 1 mg/L of 4-MBC, one of them covered by aluminium foil (blank) were kept under artificial UV radiation provided by a mercury lamp. At each 10 min a sample of each solution was taken and immediately analyzed in a UV/Vis spectrophotometer and also by HPLC-UV-DAD. And it was observed that 4-MBC suffers a quickly isomerization: the *E*-form changes to the *Z*-form (Fig.4.5). It would be expected that the concentration of the *E*-form decreases along the reaction time and that the concentration of the *Z*-form increases along the reaction time (Rodil *et al*., 2009) and this behavior was observed (Fig.4.5). In the control reaction (the reaction kept in the dark) the UV-filter did not suffers isomerization and its concentration was practically constant.

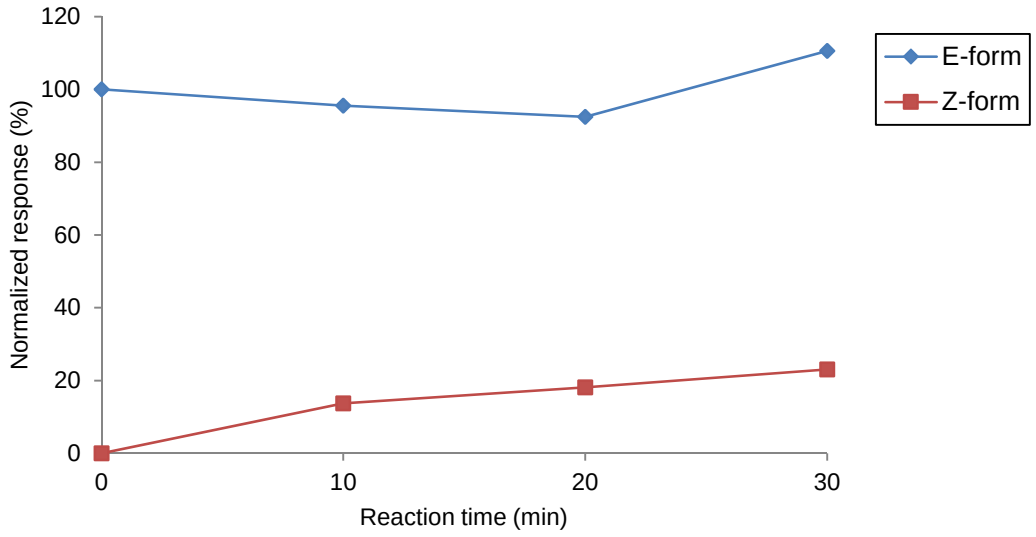

Figure 4.5. Time profiles of *E*-form and *Z*-form of 4-MBC (1 mg/L) during 30 min of artificial UV radiation treatment. Concentrations are related to the initial concentration. Results obtained by HPLC-UV-DAD.

Then, to improve the absorbance (0.04) obtained with 1 mg/L, it was tested a solution with 10 mg/L of 4-MBC. The photodegradation of this solution was studied under the same conditions of the previous one and the samples taken at 30, 40, 60 and 120 min were analyzed by UV/Vis spectrophotometry. Again, a blank solution was covered by aluminum foil and kept under the lamp. And, again, it was observed that the concentration

of 4-MBC decreased along the reaction time (Fig.4.6). In the control reaction (the reaction kept in the dark) its concentration was practically constant.

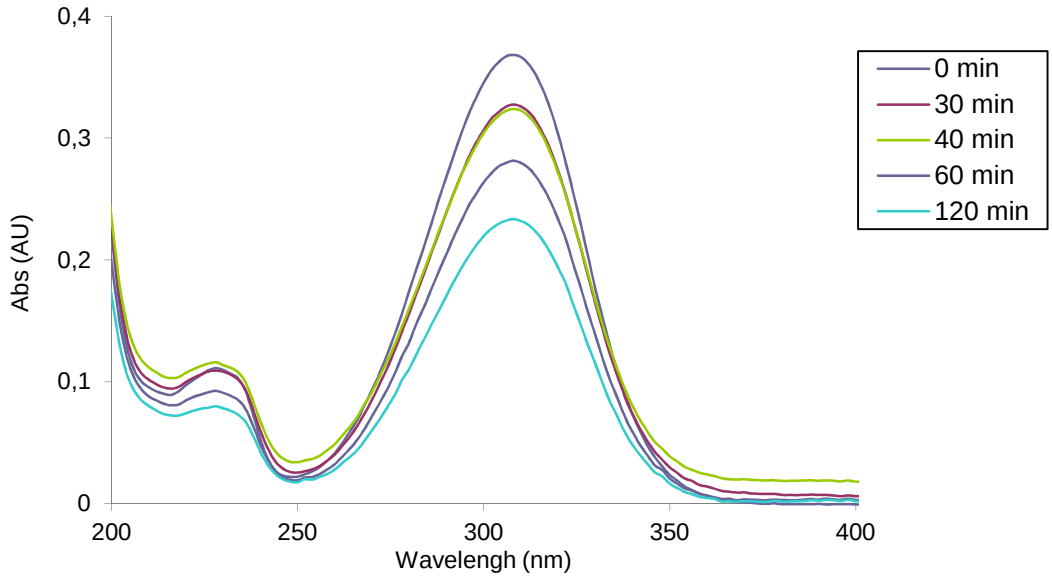

Figure 4.6. Time profiles of 4-MBC (10 mg/L) during 30 min of artificial UV radiation treatment. Results obtained by UV/Vis spectrophotometry.

The photodegradation under natural UV radiation during 30 min it was also evaluated. A solution with 1 mg/L of 4-MBC was kept under direct natural UV radiation together to another solution. As happened under artificial UV radiation, 4-MBC suffered a quickly isomerization between the two forms. And, in this case the concentration of the *E*-form decreased along the reaction time while the concentration of 4-MBC suffered an increasing (10 min) and then a decreasing (Fig.4.7). In the control reaction (the reaction kept in the dark) the UV-filter did not suffers isomerization and its concentration was practically constant.

So, it was added chlorine to evaluate if the degradation under UV radiation is higher in presence of chlorine. The reaction was carried out in glass vessels containing 100 mL of deionized water. The deionized water samples were spiked with free chlorine to get the initial concentration of 10 mg/L. After that, the pH of the aqueous solution was adjusted to 7.0 ± 0.1 and the resulting solution was then spiked with 1 mg/L of 4-MBC. This solution was kept under artificial UV radiation together to another solution with the same ratio UV-filter:chlorine covered by aluminum foil. The reaction was performed during 30 min and at each 10 min a sample of each solution was taken, the excess of chlorine was quenched with ascorbic acid according and immediately analyzed by spectrophotometric and also by HPLC-UV-DAD. The results showed that 4-MBC suffers quickly isomerization between *E*-form and *Z*-form. In presence of chlorine, the concentration of the *E*-form decreased and the concentration of the *Z*-form suffered an

increasing followed by a decreasing. Comparing to the results from the reaction without chlorine, the concentration of both isomers was higher than their concentration in presence of chlorine (Fig.4.8). In the control reaction (the reaction kept in the dark) the UV-filter did not suffers isomerization and the concentration was constant during the 30 min of the reaction.

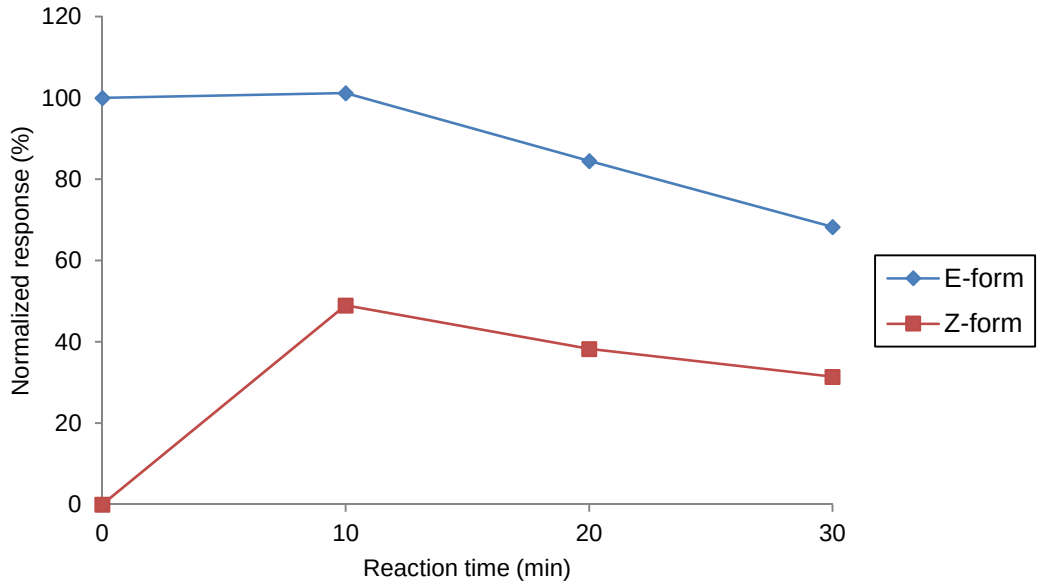

Figure 4.7. Time profiles of *E*-form and *Z*-form of 4-MBC of 4-MBC (1 mg/L) during 30 min of natural UV radiation treatment. Concentrations are related to the initial concentration. Results obtained by HPLC-UV-DAD.

Done this first approach, the photodegradation of 4-MBC was followed during a higher reaction time. The solution with 1 mg/L of 4-MBC was kept under artificial UV radiation provided by a mercury lamp. In this case, once 4-MBC did not showed degradation in the solution covered by aluminum foil, no control solution was maintained under the lamp covered by aluminum foil being that the control sample was taken at 0 min at reaction after a stirring time to ensure a good dissolution. At each 30 min a sample was taken and immediately analyzed by HPLC-UV-DAD. The isomerization of 4-MBC was only observed after 60 min of the reaction and the results related to the concentration were inconclusive. This experiment was performed with the temperature of the solution fluctuating according the increasing of the temperature caused by the heat of the lamp.

So, to minimize the effect of the temperature in the measurements, the temperature was maintained constant. A solution with 1 mg/L of 4-MBC was kept under artificial UV radiation provided by a mercury lamp. In this case, once 4-MBC did not showed degradation in the solution covered by aluminum foil, no control solution was

maintained under the lamp covered by aluminum foil being that the control sample was taken at 0 min at reaction after a stirring time to ensure a good dissolution. At each 30 min a sample was taken and immediately analyzed by HPLC-UV-DAD. The results showed that 4-MBC suffers a quickly isomerization. The concentration of the *E*-form decreased along the reaction time and the concentration of the *Z*-form increased along the reaction time (Fig.4.9).

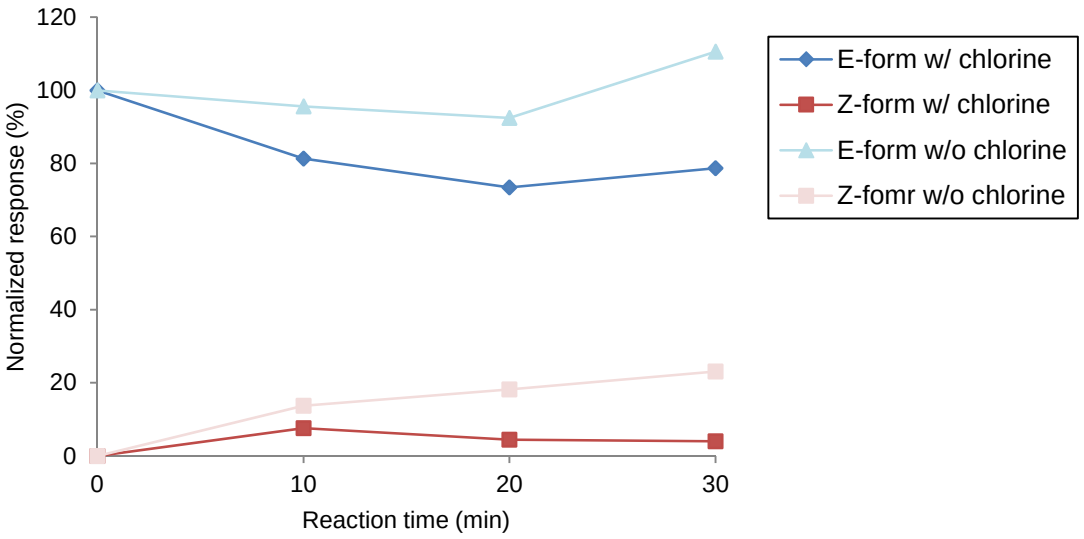

Figure 4.8. Time profiles of 4-MBC (1 mg/L) alone and in presence of chlorine (10 mg/L) during 30 min of natural UV radiation treatment. Concentrations are related to the initial concentration. Results obtained by HPLC-UV-DAD.

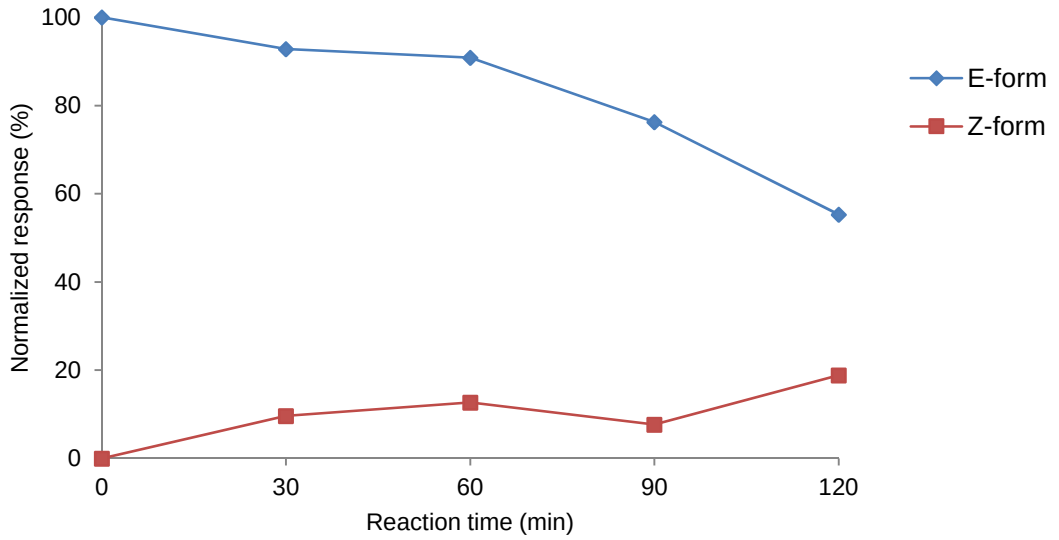

Figure 4.9. Time profiles of *E*-form and *Z*-form of 4-MBC of 4-MBC (1 mg/L) during 120 min of artificial UV radiation treatment. Concentrations are related to the initial concentration. Results obtained by HPLC-UV-DAD. Each point is the mean of two measurements.

The photodegradation of 4-MBC under natural UV radiation was also followed during a higher reaction time. A solution with 1 mg/L of 4-MBC was kept under direct natural UV radiation during 360 min and samples were taken at each 60 min. The samples were immediately analyzed by HPLC-UV-DAD. The results showed a quickly isomerization of the filter between the *E*-form and the *Z*-form with the concentration of the *E*-form decreasing and the concentration of the *Z*- form increasing slightly (Fig.4.10).

So, it was added chlorine to evaluate if the degradation under natural UV radiation is higher in presence of chlorine and this reaction was followed during 360 min. The reaction was carried out in glass vessels containing 100 mL of deionized water. The deionized water samples were spiked with free chlorine to get the initial concentration of 10 mg/L. After that, the pH of the aqueous solution was adjusted to 7.0 ± 0.1 and the resulting solution was then spiked with 1 mg/L of 4-MBC. This solution was kept under natural UV radiation. In this case, and once 4-MBC did not showed degradation in the solution covered by aluminum foil during the first approach, no control solution was performed being that the control sample was taken at 0 min at reaction after a stirring time to ensure a good dissolution (this control sample did not contained chlorine). At each 60 min a sample of the solution was taken, the excess of chlorine was quenched with ascorbic acid according and immediately analyzed by spectrophotometric and also by HPLC-UV-DAD. The results showed that 4-MBC suffers quickly isomerization. In presence of chlorine, the concentration of the *E*-form decreased and the concentration of the *Z*-form increased. Comparing to the results from the reaction without chlorine, the concentration of the *E*-form was higher than its concentration in presence of chlorine, while the concentration of the *Z*-form was similar with and without chlorine. (Fig.4.11).

Regarding the photodegradation of OC, it was performed a study very similar to the 4-MBC one. At the 30 min assay, a solution with 0.5 mg/L of OC was kept under artificial UV radiation provided by a mercury lamp. After 30 min of reaction a sample of the solution was taken and immediately analyzed by spectrophotometric and also by HPLC-UV-DAD. And it was observed that, contrary to 4-MBC, OC doesn't suffers isomerization.

Done this first approach, the photodegradation of OC was followed during a higher reaction time. A solution with 1 mg/L of OC was kept under artificial UV radiation provided by a mercury lamp during 240 min and the temperature was maintained constant. The control sample was taken at 0 min at reaction after a stirring time to ensure a good dissolution. At each 60 min a sample was taken and immediately analyzed by HPLC-UV-DAD. The results showed that the concentration of OC maintained relatively constant (Fig.4.12).

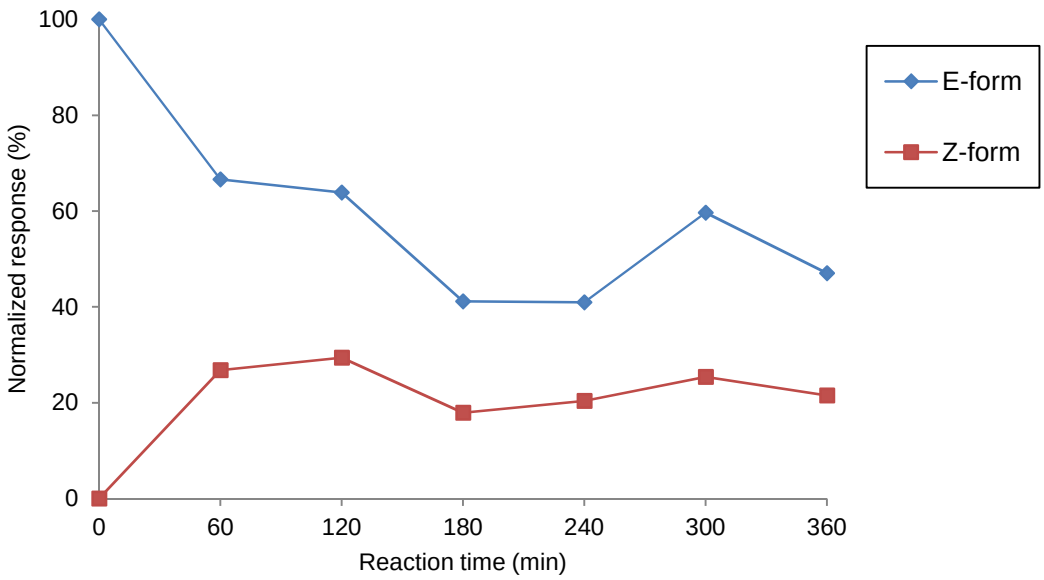

Figure 4.10. Time profiles of *E*-form and *Z*-form of 4-MBC (1 mg/L) during 360 min of natural UV radiation treatment. Concentrations are related to the initial concentration. Results obtained by HPLC-UV-DAD.

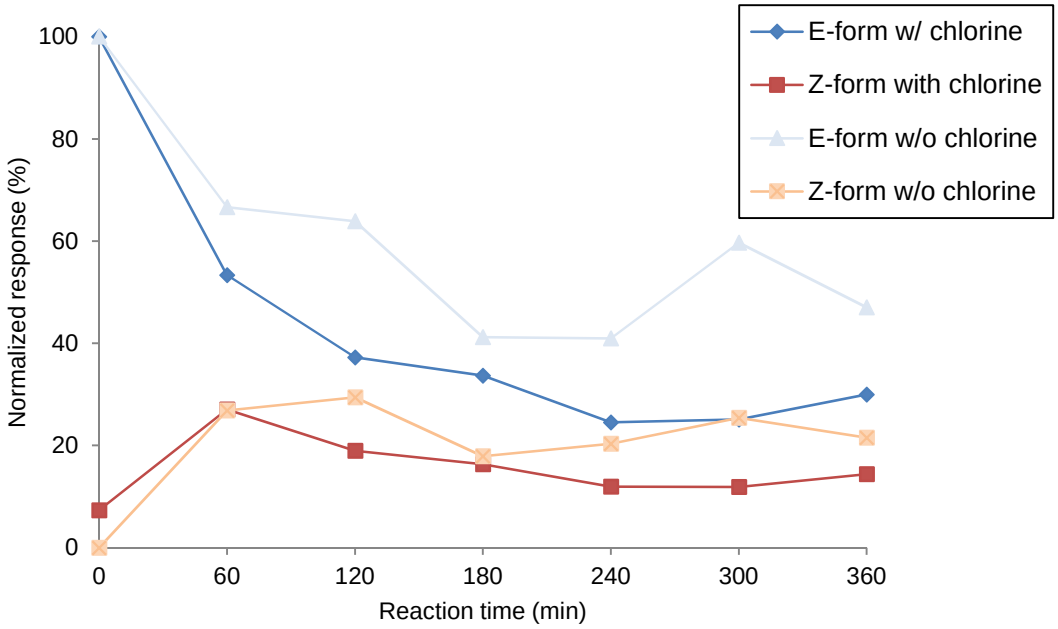

Figure 4.11. Time profiles of 4-MBC (1 mg/L) alone and in presence of chlorine (10 mg/L) during 360 min of natural UV radiation treatment. Concentrations are related to the initial concentration. Results obtained by HPLC-UV-DAD.

So, it was added chlorine to evaluate if the degradation under artificial UV radiation is higher in presence of chlorine and this reaction was followed during 240 min. The reaction was carried out in glass vessels containing 100 mL of deionized water. The

deionized water samples were spiked with free chlorine to get the initial concentration of 10 mg/L. After that, the pH of the aqueous solution was adjusted to 7.0 ± 0.1 and the resulting solution was then spiked with 1 mg/L of OC. This solution was kept under artificial UV radiation. A control solution without chlorine was kept covered by aluminum foil under artificial UV radiation. At each 60 min a sample of the reaction solution was taken, the excess of chlorine was quenched with ascorbic acid according and immediately analyzed also by HPLC-UV-DAD. Fig.4.12 compares the results obtained in this experiment and the results obtained in the reaction with no chlorine kept under artificial UV radiation.

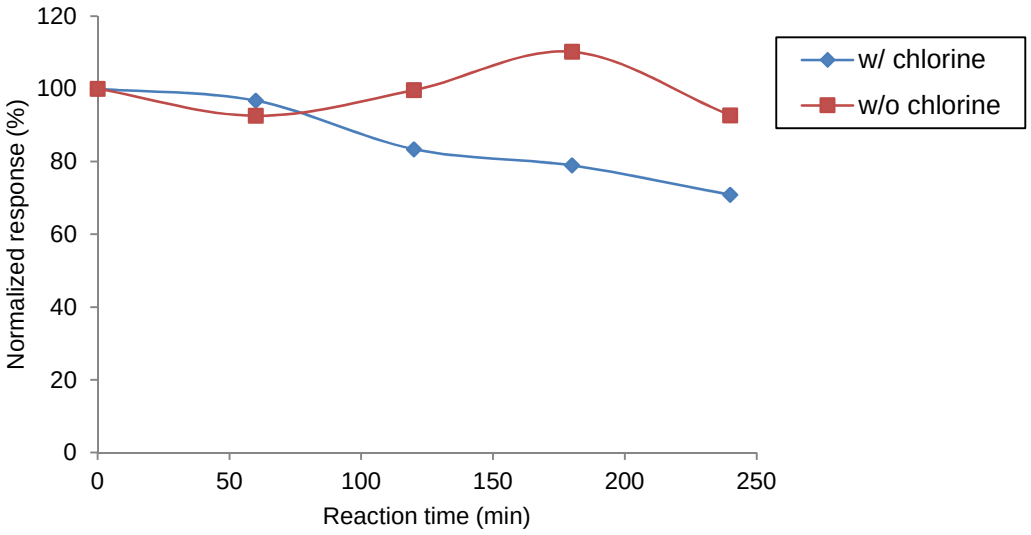

Figure 4.12. Time profiles of OC (1 mg/L) alone and in presence of chlorine (10 mg/L) during 240 min of artificial UV radiation treatment. Concentrations are related to the initial concentration. Results obtained by HPLC-UV-DAD.

The photodegradation of OC under natural UV radiation was also followed during 360 min. A solution with 1 mg/L of OC was kept under direct natural UV radiation during 360 min and samples were taken at each 60 min. The samples were immediately analyzed by HPLC-UV-DAD. The results showed that its concentration decreased along the reaction time (Fig.4.13).

So, it was added chlorine to evaluate if the degradation under natural UV radiation is higher in presence of chlorine and this reaction was followed during 360 min. The reaction was carried out in glass vessels containing 100 mL of deionized water. The deionized water samples were spiked with free chlorine to get the initial concentration of 10 mg/L. After that, the pH of the aqueous solution was adjusted to 7.0 ± 0.1 and the resulting solution was then spiked with 1 mg/L of OC. This solution was kept under natural

UV radiation and at each 60 min a sample of the solution was taken, the excess of chlorine was quenched with ascorbic acid according and immediately analyzed by HPLC-UV-DAD. Fig.4.13 compares the results obtained in this experiment and the results obtained in the reaction with no chlorine kept under artificial UV radiation.

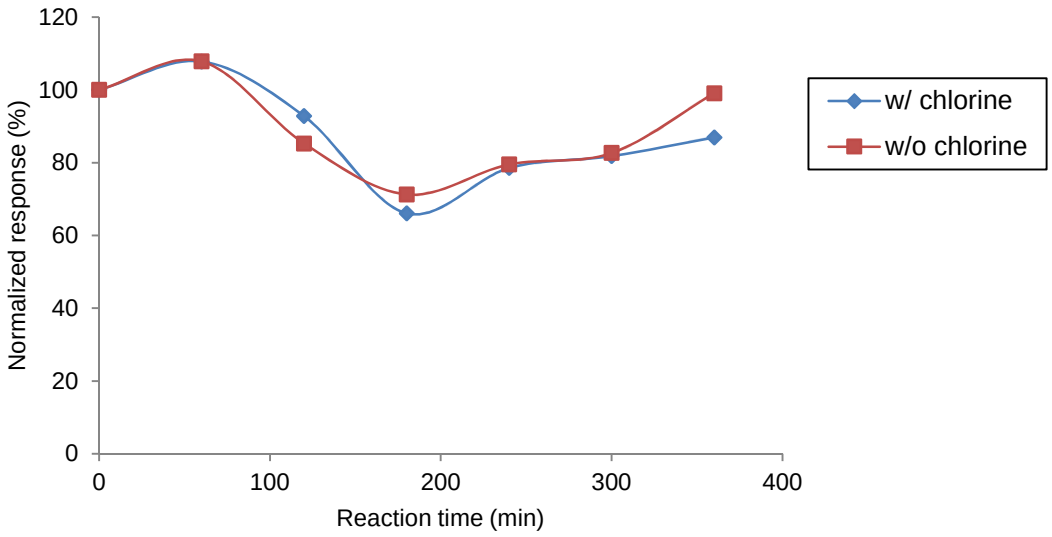

Figure 4.13. Time profiles of OC (1 mg/L) alone and in presence of chlorine (10 mg/L) during 360 min of natural UV radiation treatment. Concentrations are related to the initial concentration. Results obtained by HPLC-UV-DAD.

4.5. References

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Chapter V

Toxicological assays

5. Toxicological assays

5.1. Introduction

PPCPs have been found in several water supplies, waste waters and natural aquatic environmental around the world (Gago-Ferrero *et al.*, 2013; Subedi *et al.*, 2012; Sui *et al*., 2011; Westerhoff *et al.*, 2005). They were already detected in several animal as happened with some UV-filters found in tissue liver of Franciscana dolphin (*Pontoporia blaivinelli*) from Brazilian coast area (Gago-Ferrero *et al.*, 2013), and antihistamines, antidepressants and musks found in fishes from German Environment Species Bank (Subedi *et al.*, 2012).

UV-filters have high lipophilicity (mostly with log K_{ow} 4–8) whereby they have been shown to accumulate in the food chain and in human milk fat. However, at present, there is a scarcity of data on environmental concentrations of UV-filters (Díaz-Cruz *et al.*, 2008; Gago-Ferrero *et al.*, 2013). Moreover, concentrations reported fluctuate significantly as a function of sample location, size of the system under study (e.g., lakes and swimming pools), frequency and type of recreational activities, season of the year and hour of the day. Still, maximum concentrations reported have corresponded to mid-day on warm summer days, as expected (Díaz-Cruz *et al.*, 2008). In natural waters BP3 is the most frequently detected UV-filter with its concentrations ranging from 2 to 125 ng/L. However, is 4-MBC the UV-filter that is detected in the highest concentrations: up to 82 ng/L (Díaz-Cruz and Barceló, 2009)

Lakes river waters are the less contaminated aquatic environments. Nevertheless there are several studies describing concentrations of UV-filters in fish from rivers and lakes contaminated with wastewaters: 1.8 mg/Kg lipid (4-MBC), 2 mg/Kg lipid and 0.5 mg/Kg lipid (others UV-filters) (Díaz-Cruz and Barceló, 2009). It can be said that fishes are the primary organisms to monitor the presence of lipophilic compounds such as UV-filters (Díaz-Cruz and Barceló, 2009). OC, which also has a high lipophilicity (K_{ow} 6.88), was also already detected in tissues liver of dolphins (*Pontoporia blainvillei*) with concentrations in the range 89−782 ng/g lw and there is evidence that maternal transfer may occur through placenta and likely also through breast milk (Subedi *et al.*, 2012).

Swimming pool water had the higher levels of UV-filters. 4-MBC and BP3 were already detected in concentrations of 330 ng/L and 400 ng/L respectively (Díaz-Cruz and Barceló, 2009). In swimming pools the chlorine used in disinfection may react with the UV-filters as reacts with natural organic matter of water, yielding DBPs such as chloroform and other trihalomethanes, nitrosamines or haloacetic acids which have toxic effects like carcinogenic effects in animals and human beings (Hrudey, 2009). Now, it is mandatory to

assess the toxicity of DBPs formed from PPCPs chlorination. The knowledge of this subject is still poor but there are already a few papers published in order to study the toxicity of some of these compounds. Bladder cancer has been associated with exposure to chlorination byproducts in drinking water, and experimental evidence suggests that exposure also occurs through inhalation and dermal absorption during swimming in pools because certain DBPs have high volatility and dermal permeability. Villanueva *et al.* (Villanueva *et al.*, 2007) observed that subjects who had ever swum in a pool showed an increased risk of bladder cancer compared with those who had never swum in pools and former and current smokers present an excess risk of bladder cancer. This study also revealed a duration-response relation for cumulative time spent in swimming pools. To evaluate the genotoxicity of swimming pool water in swimmers, Kogevinas and co-workers (Kogevinas *et al.*, 2010) examined some biomarkers of genotoxicity in an experimental study in which adults swam for 40 min in a chlorinated, indoor swimming pool, comparing the biomarker results with the concentrations of four THMs (bromoform, bromodichloromethane, chloroform and chlorodibromomethane) in exhaled breath. It was observed increases in two biomarkers of genotoxicity (micronuclei in in peripheral blood lymphocytes and urinary mutagenicity). Although only brominated THMs showed genotoxicity, all four are carcinogenic in rodents.

UV-filters also showed hormonal activity *in vitro* and *in vivo* inducing vitellogenin, causing alterations in gonads, decreasing fertility and leading to the feminization in sex characteristics of male fish (Díaz-Cruz and Barceló, 2009). These effects are associated mainly with the exposure to benzophenones and camphor-related UV-filters at concentrations higher than those reported in the environment. However, the rapid accumulation of these compounds in the environment and the potential for mixture effects warrant further research to assess the fate and the effects of the UV-filters in aquatic ecosystems (Díaz-Cruz and Barceló, 2009). 4-MBC also showed effects in hypothalamuspituitary–gonadal system in male rats altering gonadal weight and steroid hormone production (Brausch and Rand, 2011).

BP3 was also found as an allergen (Berne and Ros, 1998) as well as OC which appears to be a strong allergen causing contact dermatitis in children and photoallergic contact in adults (Avenel-Audran *et al.*, 2014)

Zebrafish (*Danio* rerio) can be used as an animal model to test the accumulation and the adverse effects of UV-filters. Blüthgen *et al.* (Blüthgen *et al.*, 2014) exposed males of zebrafish to OC, which as a high tendency to bioaccumulation due to its high lipophilicity and low biodegradability. It was observed no effect on survival. Calculated bioconcentration factors [BCF = concentration in fish (ng/g body weight) / concentration in water (µg/L] ranged between 41 and 136. They also verified that OC affects transcription

of genes related to some biological pathways of G0 process in the brain (development process, anatomical structure development, system development, multicellular organismal processes end development, response to steroid hormone stimulus, anatomical structure morphogenesis, positive regulation of cellular process, organ development and cellular component organization) and in the liver (xenobiotic metabolic processes, cellular response to xenobiotic stimulus, urea cycle and metabolic processes, nitrogen cycle metabolic processes, lung and respiratory tube development ad response to vitamin B2). Transcription of genes related to pathways responsible for fat cell differentiation, regulation of the metabolism of thyroide hormones (triiodothyronine and thyroxine) and thyroxine signaling, polyamine metabolism (polyamines are important to DNA replication and consequently to cell growth), inflammatory mediators signaling, cytoskeleton intermediate filaments, and signal transduction of the androgen nuclear receptor pathway and others are also affected by the exposure to OC.

The products from photodegradation of UV-filters may also cause some toxic concerns (Butt and Christensen, 2000). Photodegradation of BDM and EHMC is already known and the toxicity of their photoproducts was already studied using cells of mouse lymphoma by Butt and Christensen (Butt and Christensen, 2000). It was observed that the parental compound of EHMC caused cell death as well as the parental compound of BDM, although this last one is less toxic since it was necessary a higher concentration to cause cell death. Regarding photoproducts, in this study only EHMC showed products resulting from its photodegradation which were more toxic than the parental compound.

BDM is a commonly used UV-filter present in many sunscreens absorbing in UVA region (Shaath, 2010). The chlorination reaction of BDM was already assessed by Santos *et al*. (Santos *et al*., 2013) and its DBPs were already identified as mono- and dichloro-substituted compounds resulting from substitution of the hydrogen atoms in the benzene rings by one or two chlorine atoms. So, the aim of this study was given a first approach on the assessment of the toxicity of the UV-filter BDM and its DBPs.

5.2. Materials and Methods

5.2.1. Reagents

BDM, CAS Registry No. [70356-09-1], 98%, (molecular formula $C_{20}H_{22}O3$) was purchased from Merck. A commercial sodium hypochlorite solution with a chlorine content

of <5% (28 mg/L of free chlorine) was used to obtain DBPs from BDM. This solution was stored at 4ºC and its free chlorine content was periodically measured by a small compact photometer for chlorine (HANNA Checker Mini Hi 701 series). Stock solutions of the BDM were prepared with methanol from Merck. Deionized water (conductivity < 0.1 μS cm^{-1}) was used in all experiments. Ascorbic acid (99.7%) used to quench the free chlorine was obtained from Merck. Sodium chlorine used to enrich the solutions was obtained from José M. Valz Pereira, LDA., Lisboa, Portugal. The pH of the solutions was adjusted to a pre-determined value with HCl solutions 0.1 mol/L. Eluents for chromatographic analysis (methanol) were liquid chromatographic grade and were bought from Merck. A Nikon Eclipse TE300 microscope and a VWR stereo microscope SZT series were used to the daily readings.

5.2.2. Zebrafish and egg production

Zebrafish (*Danio rerio*) larvae were acutely exposed to several concentrations of BDM and corresponding DBPs solution resulting from chlorination reaction. The effect of these compounds regarding mortality and anatomic abnormalities was assessed.

5.2.2.1. Parental generation

Adult wild-type zebrafish (*Danio rerio*) were obtained from Singapore and were used as breeding stocks. Zebrafish specimens were kept in 250 L aquaria with dechlorinated water in a recirculation system with mechanical filters at a temperature water of (28 ± 1)ºC on a photoperiod 14:10 h (light:dark). The fishes were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany). The aquaria contained 12-14 females and 6-7 males.

5.2.2.2. Egg production of parental generation

In the afternoon before breeding, two groups of zebrafish couples were independently housed in cages attached to the aquaria with water circulating between the aquaria and the cages (one couple per aquarium). The photoperiod conditions were also the same and in this day the fishes were fed *ad libitum* four times. At the morning of the following day, breeding fish were removed 1.5 h after the beginning of the light period and

the eggs were collected and cleaned. This point was recorded as 0 h post fertilization (hpf). Fertilized eggs were allocated in petri dish with autoclaved water and methylene blue kept at (28 ± 1)ºC on a photoperiod 14:10 h (light:dark).

5.2.3. Exposure (study design)

Three days post fertilization (dpf) the larvae were collected and exposed to the solutions. Four assays were performed separately and the exposure conditions were different.

5.2.3.1. First assay

Larvae were randomly distributed in 6-well plate (5 larvae per exposure condition; 400 µL solution/well) and kept at (28 ± 1)ºC on a photoperiod 14:10 h (light:dark) throughout the assay. Larvae were continuously exposed to the exposure conditions from 3 dpf to 5 dpf. In this assay, BDM and its DBPs were applied at the concentration of 1 mg/L. Stock solutions of BDM in methanol (about 100 mg/L) were first prepared. The tested solutions were prepared in deionized water spiked with the stock solution. The DBPs were obtained by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L). This reaction was performed at the beginning of each day whereby the DBPs solutions contained some chlorine. The concentration of the DBPs was determined by HPLC-UV-DAD comparing the pikes corresponding to the same concentration of the parental BDM. A solution of deionized water spiked with free chlorine to get the concentration of 10 mg/L was bubbled with air to remove the chlorine was used as control. At the beginning of the assay, the chlorine concentration in the control solution was 0.04 ppm. All the solutions were kept in the dark till they were applied. Dead larvae were removed during the daily readings and two out of three of each well content were renewed with freshly prepared exposure solution. Potential anatomic abnormalities were also recorded.

5.2.3.2. Second assay

Larvae were randomly distributed in 24-well plate (5 larvae per well, 15 larvae per exposure condition; 500 µL solution/well) and kept at (28 ± 1)ºC on a photoperiod 14:10 h

(light:dark) throughout the assay. Larvae were continuously exposed to the exposure conditions from 3 dpf to 6 dpf. In this assay, BDM and its DBPs were applied at the concentration of 1 mg/L. Stock solutions of BDM in methanol (about 100 mg/L) were first prepared. The tested solutions were prepared in deionized water enriched with sodium chloride (NaCl) to get the concentration of 5.03 mM (CSH protocols, 2011) and spiked with the stock solution. The DBPs were obtained by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L). This reaction was performed 3 days before the application, with the purpose to get the concentration of free chlorine of 0.30 mg/L. The concentration of the DBPs was determined by HPLC-UV-DAD comparing the pikes corresponding to the same concentration of the parental BDM. A solution of deionized water spiked with free chlorine to get the concentration of 0.3 mg/L, a solution of deionized water spiked with free chlorine at the concentration of 0.3 mg/L enriched with NaCl (to get the concentration of 5.03 mM) and a solution of deionized water with 5.03 mM of NaCl were used as control. Autoclaved water was also used as control. All the solutions were kept in the dark till they were applied. Dead larvae were removed during the daily readings and two out of three of each well content were renewed with freshly prepared exposure solution. Potential anatomic abnormalities were also recorded.

5.2.3.3. Third assay

Larvae were randomly distributed in 24-well plate (15 larvae per well, 30 larvae per exposure condition; 500 µL solution/well) and kept at (28 ± 1)ºC on a photoperiod 14:10 h (light:dark) throughout the assay. Larvae were continuously exposed to the exposure conditions from 3 dpf to 7 dpf. In this assay, BDM and its DBPs were applied at the concentration of 0.1 mg/L. Stock solutions of BDM in methanol (about 100 mg/L) were first prepared. The tested solutions were prepared in deionized water enriched with sodium chloride (NaCl) to get the concentration of 5.03 mM (CSH protocols, 2011) and spiked with the stock solution. The DBPs were obtained by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L). After 30 min of the beginning of this reaction (this reaction time assure that BDM is totally transformed by chlorine) the solution was concentrated by liquid-liquid extraction with ethyl acetate (three extractions with 20 mL each). After this procedure the organic solvent was evaporated and 1 mL of methanol was added to the final residue. The concentration of the DBPs was determined by HPLC-UV-DAD comparing the pikes corresponding to the same concentration of the parental BDM. These samples were maintained in dark until they were used and dilute in deionized water enriched with NaCl. Although this procedure assured that almost all of the free chlorine

was eliminated, free chlorine content of the final solutions of DBPs was assessed and, when it was necessary, it was added ascorbic acid to neutralize free chlorine. So, a solution with ascorbic acid at the concentration of 0.15 mg/L (the concentration requested to neutralize the higher level of free chlorine that was found) was used as control. A solution of deionized water with 5.03 mM of NaCl and autoclaved water were also used as controls. All the solutions were kept in the dark till they were applied. Dead larvae were removed during the daily readings and two out of three of each well content was renewed with freshly prepared exposure solution. Potential anatomic abnormalities were also recorded.

5.2.3.4. Fourth assay

Larvae were randomly distributed in 24-well plate (5 larvae per well, 15 larvae per exposure condition; 500 µL solution/well) and kept at (28 ± 1)ºC on a photoperiod 14:10 h (light:dark) throughout the assay. Larvae were continuously exposed to the exposure conditions from 3 dpf to 7 dpf. In this assay, BDM and its DBPs were applied at the concentration of 0.1, 1 and 10 µg/L. Stock solutions of BDM in methanol (about 100 mg/L) were first prepared. The tested solutions were prepared in deionized water enriched with sodium chloride (NaCl) to get the concentration of 5.03 mM (CSH protocols, 2011) and spiked with the stock solution. The DBPs were obtained by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L). After 30 min of the beginning of this reaction (this reaction time assure that BDM is totally transformed by chlorine) the solution was concentrated by liquid-liquid extraction with ethyl acetate (three extractions with 20 mL each). After this procedure the organic solvent was evaporated and 1 mL of methanol was added to the final residue. The concentration of the DBPs was determined by HPLC-UV-DAD comparing the pikes corresponding to the same concentration of the parental BDM. These samples were maintained at -4ºC and in dark until they were used and dilute in deionized water enriched with NaCl. Although this procedure assured that almost all of the free chlorine was eliminated, free chlorine content of the final solutions of DBPs was assessed and, when it was necessary, it was added ascorbic acid to neutralize free chlorine. So, a solution with ascorbic acid at the concentration of 0.15 mg/L (the concentration requested to neutralize the higher level of free chlorine that was found) was used as control. A solution of deionized water with 5.03 mM of NaCl and autoclaved water were also used as controls. All the solutions were kept in the dark till they were applied. Dead larvae were removed during the daily readings and two out of three of each well

content were renewed with freshly prepared exposure solution. Potential anatomic abnormalities were also recorded.

5.2.3.5. Touch response test

At the end of the third assay, with the larvae with 7 dpf, it was performed a touch response test. At 96 hpf the larva is freely swimming and is able to change swimming directions spontaneously and to direct its swimming towards targets. Mechanical stimuli near the head of the larva induce a fast escape response (Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva). Tests like this on allow the identification of changes in sensory and locomotor functions (The Developmental Neurotoxicity of Fipronil: Notochord Degeneration and Locomotor Defects in Zebrafish Embryos and Larvae). After the last reading of the third assay, each larva was displaced to a petri dish with autoclaved water. After a little period of habituation and stabilization (around 2 min), it was applied a short mechanical stimulus with a pipette tip in its head and tail. It was recorded the response of the larvae regarding escaping or don't.

5.2.4. Statistics

All the statistical tests were performed in Microsoft Excel 2013.

5.3. Results and discussion

Zebrafish (*Danio rerio*) was used to assess the toxicity caused by another UV-filter already studied: BDM (Santos *et al*., 2013). Larvae of Zebrafish were acutely exposed to several concentrations of BDM and corresponding DBPs solution resulting from chlorination reaction. The effect of these compounds regarding mortality and anatomic abnormalities was assessed.

5.3.1. First assay

Larvae were randomly distributed in 6-well plate (5 larvae per exposure condition; 400 µL solution/well) and kept at (28 ± 1)ºC on a photoperiod 14:10 h (light:dark)

throughout the assay. Larvae were continuously exposed to 1 mg/L of BDM and 1 mg/L of its DBPs from 3 dpf to 5 dpf. In this assay, the control solution was deionized water spiked with free chlorine to get the concentration of 10 mg/L and bubbled with air to remove the chlorine. At the beginning of this assay, the chlorine level was 0.04 ppm.

The DBPs were obtained by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L). This reaction was performed at the beginning of each day whereby the DBPs solutions contained some chlorine. Before all these toxicological tests, the behavior of the DBPs was followed by HPLC-MS and the pikes obtained in the chromatogram were consistent to the pikes obtained by Santos *et al.* (Santos *et al*., 2013). After 30 min of reaction BDM is completely transformed and the concentration of its DBPs reaches its maximum. After that, it was observed that the concentration of the DBPs decreases as the time goes by, as well as the concentration of free chlorine. However, at the beginning of this assay, the concentration of chlorine was too high that all the larvae exposed to the solution of DBPs died after the first day of the assay. All the larvae exposed to BDM showed abnormalities and the larvae in the control also showed mortality and some abnormalities

So, this first assay was finished after the first day of exposure and the solutions of the exposure conditions were improved to others assays.

5.3.2. Second assay

Larvae were randomly distributed in 24-well plate (5 larvae per well, 15 larvae per exposure condition; 500 µL solution/well) and kept at (28 ± 1)ºC on a photoperiod 14:10 h (light:dark) throughout the assay. Larvae were continuously exposed to 1 mg/L of BDM and 1 mg/L of its DBPs from 3 dpf to 6 dpf. In this assay, two different solutions of DBPs were tested. In the first one, similar to the first assay, the DBPs were obtained by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L) and this reaction was performed at the beginning of each day. So, these DBPs solutions contained high levels of free chlorine. In the second one, and since the behavior of the DBPs and free chlorine concentration along the reaction time was followed, the DBPs were obtained also by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L) but it was applied to the larvae only after 3 days, when the concentration of free chlorine is lower (0.30 ppm).

In the first assay, the higher mortality observed may be due to the fact that the tested solutions did not contain any nutrients necessary to a correct development of the larvae since the tested solutions were prepared with deionized water. So, to overcome

this question, at the beginning of the assay the tested solutions were applied with the double of the concentration diluted in the zebrafish medium (50:50 of tested solution:zebrafish medium). In the next days, the tested solutions were enriched with NaCl to provide the nutritional requirement of the larvae. So, the control solutions were autoclaved water (which was the zebrafish medium), deionized water with free chlorine ate 0.30 ppm, deionized water with NaCl and deionized water with free chlorine (0.30 ppm) enriched with NaCl.

In the end of this assay (larvae at 6 dpf) the larvae exposed to BDM showed neither mortality (Fig.5.1) nor abnormalities (Fig.5.2). However, the larvae exposed to the BDM solution with 0.30 ppm of chlorine showed some abnormalities (Fig.5.2) at the end of the assay. These abnormalities consisted in skeletal defects (curved spine) but these results don't have statistical significance ($p>0.05$). All the larvae exposed to BDM solution with 10 mg/L of chlorine died after the first day of the assay.

Since the lack of nutrients may be the reason to the high mortality during the first assay, here it was compared two control solutions: autoclaved water and deionized water enriched with NaCl. No mortality was observed in these two tested conditions (Fig.5.1) however, at the end of this assay (larvae at 6 dpf), the larvae in autoclaved water showed skeletal defects (Fig.5.2) which don't have statistical significance ($p>0.05$).

To assess the effect the 0.30 ppm of free chlorine, it was compared two others control solutions: deionized water with free chlorine ate 0.30 ppm and deionized water with free chlorine (0.30 ppm) enriched with NaCl. No mortality was recorded in any of these two tested conditions (Fig.5.1). However, at the end of this assay (larvae at 6 dpf), the larvae exposed to the solution with free chlorine enriched with NaCl showed skeletal defects (Fig.5.2) which don't have statistical significance ($p>0.05$). Still, at the third day of the assay (larvae at 5 dpf) the number of larvae exposed to the solution with free chlorine enriched with NaCl with skeletal defects was higher than the number recorded at the end of the assay (larvae at 6 dpf) (Fig.5.3), whereby the larvae showed some kind of regeneration. Similar to the previous result, these effects don't have statistical significance ($p>0.05$).

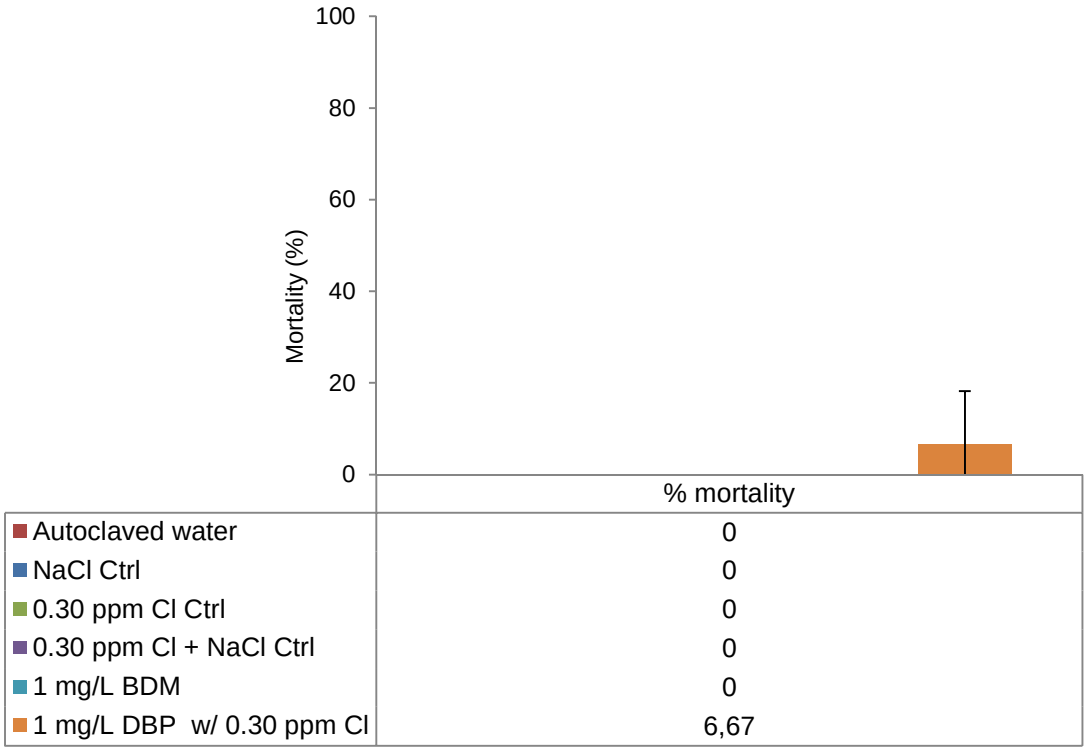


Figure 5.1. Percentage of mortality at the end of the assay (6 dpf) for the four control solutions (autoclaved water, deionized water enriched with NaCl, deionized water spiked with chlorine to get 0.30 ppm of free chlorine, and deionized water spiked with chlorine to get 0.30 ppm of free chlorine enriched with NaCl), 1 mg/L of BDM and 1 mg/L of DBPs with 0.30 ppm of chlorine.

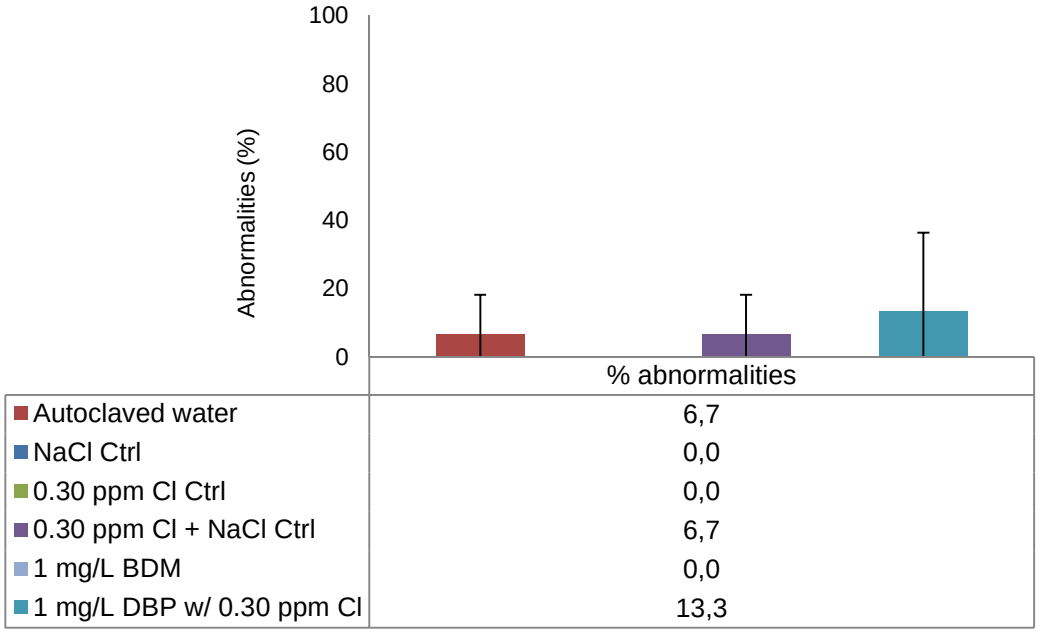


Figure 5.2. Percentage of abnormalities at the end of the assay (6 dpf) for the four control solutions (autoclaved water, deionized water enriched with NaCl, deionized water spiked with chlorine to get 0.30 ppm of free chlorine, and deionized water spiked with chlorine to get 0.30 ppm of free chlorine enriched with NaCl), 1 mg/L of BDM and 1 mg/L of DBPs with 0.30 ppm of chlorine.

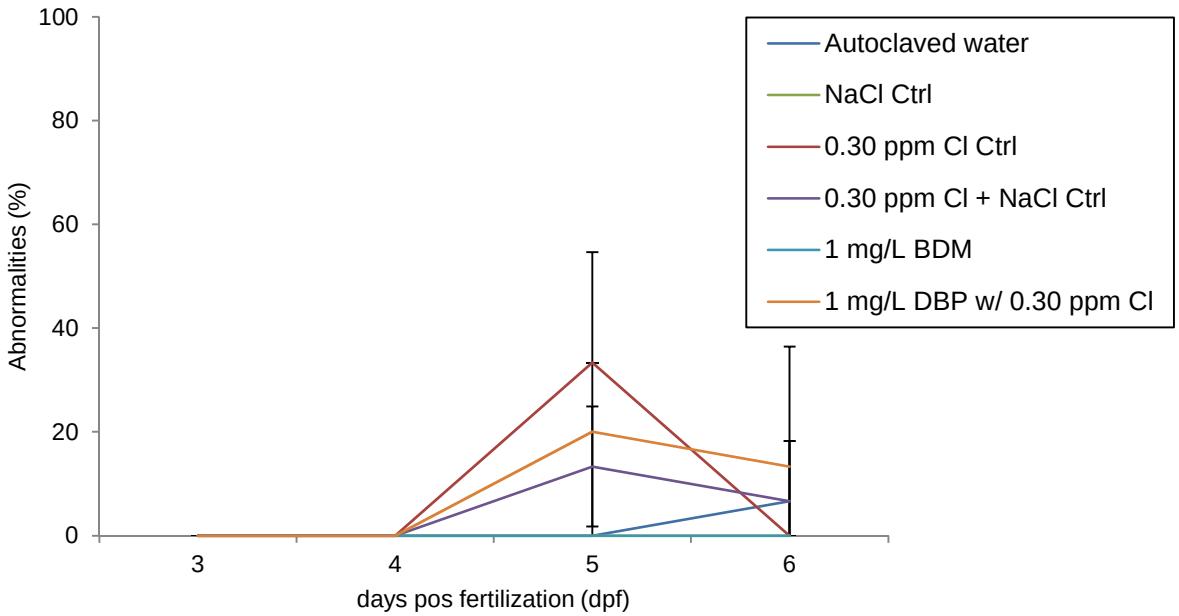


Figure 5.3. Percentage of abnormalities along the assay for the four control solutions (autoclaved water, deionized water enriched with NaCl, deionized water spiked with chlorine to get 0.30 ppm of free chlorine, and deionized water spiked with chlorine to get 0.30 ppm of free chlorine enriched with NaCl), 1 mg/L of BDM and 1 mg/L of DBPs with 0.30 ppm of chlorine.

So, due to the lack of statistical relevance of this assay, it cannot be concluded if the parental compound on larvae as different effects than the ones caused by its DBPs.

5.3.3. Third assay

Larvae were randomly distributed in 24-well plate (15 larvae per well, 30 larvae per exposure condition; 500 µL solution/well) and kept at (28 ± 1)ºC on a photoperiod 14:10 h (light:dark) throughout the assay. Larvae were continuously exposed to 0.1 mg/L of BDM and 0.1 mg/L of its DBPs from 3 dpf to 7 dpf. In this assay, two different solutions of DBPs were tested. In the first one, similar to the others assay, the DBPs were obtained by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L). In this case, this reaction was performed at 12h before the each day of the assay. After 30 min of reaction (this reaction time assures that BDM is totally transformed by chlorine) the solution was concentrated by liquid-liquid extraction with ethyl acetate and the organic solvent was evaporated. These procedure ensured that the chlorine present in the solutions was removed, eliminating this variable in the assay. Nevertheless, the chlorine level of the solution of DBPs was controlled as well as the pH (pH near 7). Whenever

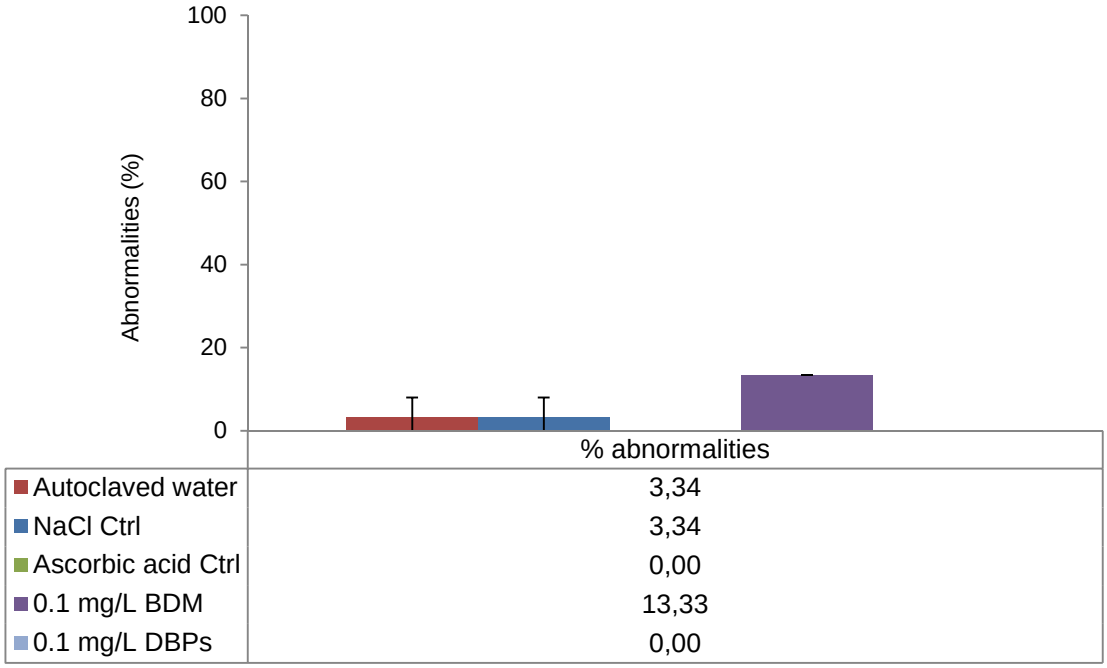
there was free chlorine in the solutions, it was added ascorbic acid to neutralize it. The behavior of the DBPs in these solutions was followed by HPLC-UV-DAD and it was observed that the concentration of the DBPs decreased along the time, whereby the solutions of DBPs were prepared daily.

To ensure the nutritional requirement of the larvae, it was maintained the same procedure of the second assay: at the beginning of the assay the tested solutions were applied with the double of the concentration diluted in the zebrafish medium (50:50 of tested solution:zebrafish medium). In the next days, the tested solutions were enriched with NaCl to provide the nutritional requirement of the larvae. So, the control solutions were autoclaved water (which was the zebrafish medium), and deionized enriched with NaCl. Once there was the risk that chlorine was not completely removed during the extraction process, a solution of deionized water and ascorbic acid (0.15 ppm, the minimum value of ascorbic acid necessary to neutralize the free chlorine; this value was obtained during preliminary experiments) was also applied as control.

In the end of this assay (larvae at 7 dpf) the larvae exposed to BDM showed no mortality but it was recorded some skeletal defects (curved spine): 13.33% of the larvae exposed to this exposure condition (Fig.5.4). However, comparing to the control (deionized water enriched with NaCl) these results don't have statistical relevance (p>0.05). Similar to the previous assay, at the fourth day of the assay (larvae at 6 dpf) the percentage of larvae exposed to the BDM with skeletal defects (60%) was higher than the percentage recorded at the end of the assay (larvae at 7 dpf) (Fig.5.5), whereby the larvae showed some kind of regeneration. This result was statistical relevant (p<0.05).

Regarding the larvae exposed to the DBPs, it was observed no mortality but it was observed skeletal defects (curved spine): 3.33 % of the larvae at the end of the second day of the assay (larvae at 4 dpf) but this result was not statistical relevant (p>0.05) comparing to the control (deionized water with ascorbic acid enriched with NaCl). In the rest of the assay, the larvae apparently recovered from these defects (Fig.5.5).

In the controls, it was observed a larva with a hemorrhage in the autoclaved water since the beginning of the assay. This larva survived until the end. In deionized water enriched with NaCl, 3.33 % of the larvae showed skeletal defects (curved spine) since 5 dpf and no effect caused by ascorbic acid in the corresponding control was detected. These differences between these two controls are not statistical relevant (p>0.05) (Fig.5.4).



	% abnormalities
Autoclaved water	3,34
NaCl Ctrl	3,34
Ascorbic acid Ctrl	0,00
0.1 mg/L BDM	13,33
0.1 mg/L DBPs	0,00

Figure 5.4. Percentage of abnormalities at the end of the assay (7 dpf) for the three control solutions (autoclaved water, deionized water enriched with NaCl, ascorbic acid enriched with NaCl), 0.1 mg/L of BDM and 0.1 mg/L of DBPs.

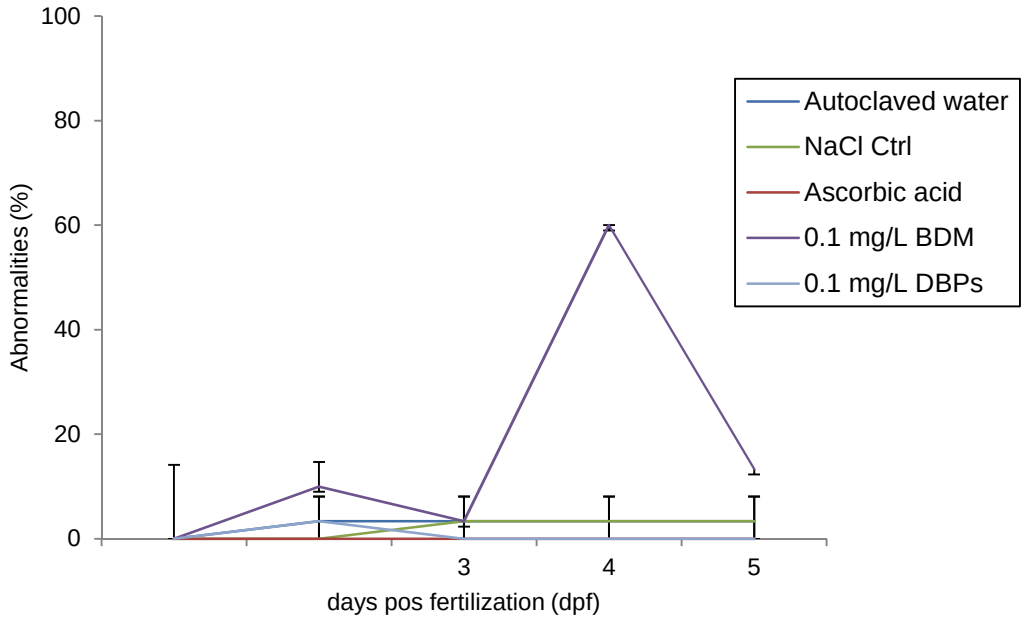


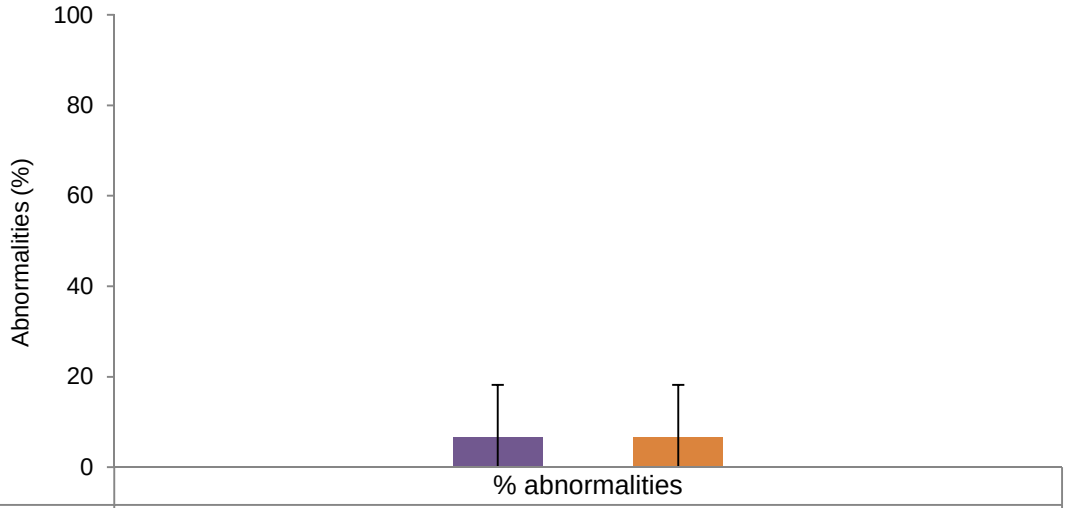
Figure 5.5. Percentage of abnormalities along the assay for the three control solutions (autoclaved water, deionized water enriched with NaCl, ascorbic acid enriched with NaCl), 0.1 mg/L of BDM and 0.1 mg/L of DBPs.

So, from this assay, it can be concluded that BDM causes abnormalities to the larvae of zebrafish at the concentration of 0.1 mg/L but its DBPs at the same concentration have no effect on the larvae.

5.3.4. Fourth assay

Larvae were randomly distributed in 24-well plate (5 larvae per well, 15 larvae per exposure condition; 500 µL solution/well) and kept at (28 ± 1)ºC on a photoperiod 14:10 h (light:dark) throughout the assay. Larvae were continuously exposed to 0.1 µg/L, 1 µg/L and 10 µg/L of BDM and 0.1 µg/L, 1 µg/L and 10 µg/L of its DBPs from 3 dpf to 7 dpf. In this assay, two different solutions of DBPs were tested. In the first one, similar to the others assay, the DBPs were obtained by the reaction between BDM and free chlorine (ratio UV-filter:chlorine 1:10 mg/L). In this case, this reaction was performed several days before the each day of the assay and the concentrated samples were kept at -4ºC. After 30 min of reaction (this reaction time assures that BDM is totally transformed by chlorine) the solution was concentrated by liquid-liquid extraction with ethyl acetate and the organic solvent was evaporated. These procedure ensured that the chlorine present in the solutions was removed, eliminating this variable in the assay. Nevertheless, the chlorine level of the solution of DBPs was controlled as well as the pH (pH near 7). Whenever there was free chlorine in the solutions, it was added ascorbic acid to neutralize it. Still, in this assay it was not necessary add ascorbic acid to the DBPs solutions. The behavior of the DBPs kept in ice was followed by HPLC-UV-DAD and it was observed that the concentration of the DBPs decreased along the time but not so fast as it happens at the temperature room, whereby the solutions of DBPs were prepared several days before the beginning of the assay. However, every day of the assay, new solutions of DBPs were prepared to minimize the decrease of its concentration.

To ensure the nutritional requirement of the larvae, it was maintained the same procedure of the second assay: at the beginning of the assay the tested solutions were applied with the double of the concentration diluted in the zebrafish medium (50:50 of tested solution:zebrafish medium). In the next days, the tested solutions were enriched with NaCl to provide the nutritional requirement of the larvae. So, the control solutions were autoclaved water (which was the zebrafish medium), and deionized enriched with NaCl. Once there was the risk that chlorine was not completely removed during the extraction process, a solution of deionized water and ascorbic acid (0.15 ppm, the minimum value of ascorbic acid necessary to neutralize the free chlorine. This value was obtained during preliminary experiments) was also applied as control.



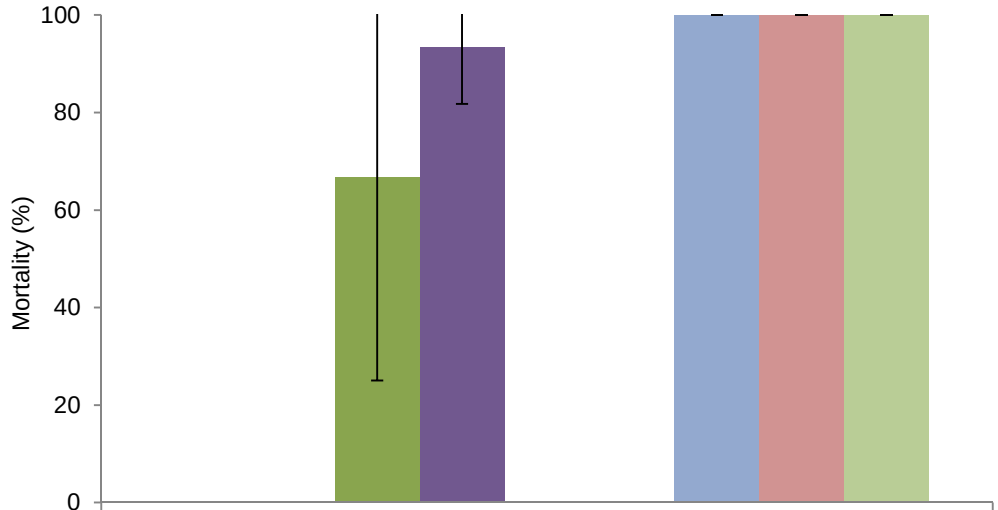
	% abnormalities
Autoclaved water	0,00
NaCl Ctrl	0,00
Ascorbic acid	0,00
0.1 ug/L BDM	6,67
1 ug/L BDM	0,00
10 ug/L BDM	6,67
0.1 ug/L DBPs	0,00
1 ug/L DBPs	0,00
10 ug/L DBPs	0,00

Figure 5.6. Percentage of mortality at the end of the assay (7 dpf) for the three control solutions (autoclaved water, deionized water enriched with NaCl, ascorbic acid enriched with NaCl), 0.1 µg /L, 1 µg/L and 10 µg/L of BDM, and 0.1 µg /L, 1 µg/L and 10 µg/L of DBPs.

In the end of this assay (larvae at 7 dpf) the larvae exposed to 0.1 µg/L of BDM showed 93.3 % of mortality (Fig.5.6). Comparing to the control (deionized water enriched with NaCl) these results have statistical relevance (p<0.05). 6.67 % of these larvae showed an abnormal position swimming with the side facing down (Fig.5.7). However, comparing to the control (deionized water enriched with NaCl) these results don't have statistical relevance (p>0.05). The larvae exposed to 1 µg/L and to 10 µg/L of BDM showed no mortality and none abnormalities.

In the end of this assay (larvae at 7 dpf) the larvae of the three experimental conditions (0.1 µg/L, 1 µg/L and 10 µg/L of DBPs) showed 100 % of mortality (Fig.5.6) and these results have statistical relevance (p<0.05). It was observed that the larvae exposed to 10 µg/L died at the second day of the assay (4 dpf) (Fig.5.8). At this point, the larvae exposed to 1 µg/L of DBPs have already 80 % of mortality (p<0.05) and the larvae exposed to 0.1 µg/L of DBPs don't have mortality although they showed 33.3 % of some abnormalities: 26.6 % of heart failure, and 6.6 % of the larvae showed an abnormal position swimming with the side facing down. However, comparing to the control

(deionized water enriched with NaCl) these results don't have statistical relevance (p>0.05). At the third day of the assay (5 dpf), the conditions of the larvae exposed to 1 µg/L of DBPs didn't suffer any change, while the mortality of the larvae exposed to 0.1 µg/L increased to 33.3 % and 26.6 % showed an abnormal position swimming with the side facing down. The dead larvae were those were with heart failure at 4 dpf. However, these results don't have statistical relevance (p>0.05) comparing to the control (deionized water enriched with NaCl). At the fourth day of the assay (6 dpf), the mortality of the larvae exposed to 1 µg/L increased to 80 % (p<0.05) and it was observed none abnormalities. The mortality of the larvae exposed to 0.1 µg/L was the same and the percentage of abnormalities increased to 33.3 % (p>0.05).



	% mortality
Autoclaved water	0,00
NaCl Ctrl	0,00
Ascorbic acid	66,67
0.1 ug/L BDM	93,33
1 ug/L BDM	0,00
10 ug/L BDM	0,00
0.1 ug/L DBPs	100,00
1 ug/L DBPs	100,00
10 ug/L DBPs	100,00

Figure 5.7. Percentage of abnormalities at the end of the assay (7 dpf) for the three control solutions (autoclaved water, deionized water enriched with NaCl, ascorbic acid enriched with NaCl), 0.1 µg /L, 1 µg/L and 10 µg/L of BDM, and 0.1 µg /L, 1 µg/L and 10 µg/L of DBPs.

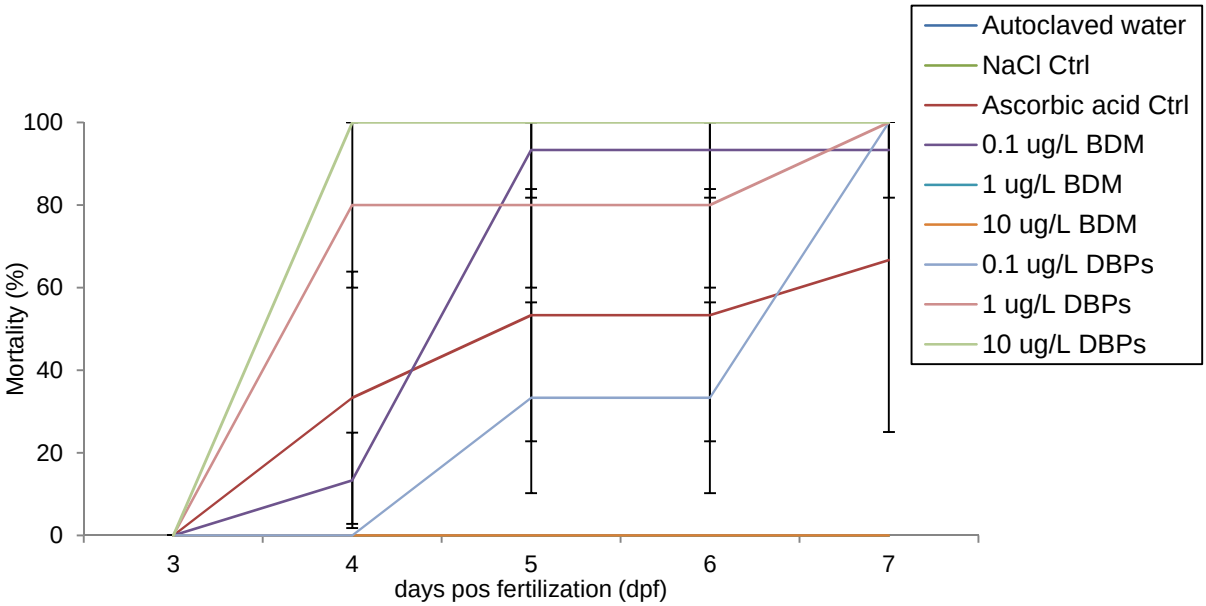


Figure 5.8. Percentage of mortality along the assay for the three control solutions (autoclaved water, deionized water enriched with NaCl, ascorbic acid enriched with NaCl), 0.1 µg /L, 1 µg/L and 10 µg/L of BDM, and 0.1 µg /L, 1 µg/L and 10 µg/L of DBPs.

Comparing the effects caused by BDM and the effects caused by the DBPs it was observed significant differences (p<0.05) between the results at the end of the assay. And from this assay, it can be concluded that BDM causes mortality on the larvae of zebrafish at the concentration of 0.1 µg/L, at the concentration of 10 µg/L DMB has no effect on the larvae of zebrafish, and its DBPs causes mortality at the three tested concentrations: 0.1 µg/L, 1 µg/L and 10 µg/L.

5.3.4. Touch response test

At the end of the third assay, with the larvae with 7 dpf, it was performed a touch response test.

Embryos of zebrafish exhibit the first movements at 17 hpf. These movements consist of alternating side-to-side contractions of the tail that curls slowly whereby the tip of the tail reaches de head. At 24 hpf the larvae react already to mechanical stimuli on the head or the tail by contracting the tail in a faster way than they already do at 17 hpf. At 26 hpf the larvae of zebrafish acquire the ability to swim when it is applied a mechanical stimulus (Saint-Amant and Drapeau, 1998). At 96 hpf the larva is freely swimming and is able to change swimming directions spontaneously and to direct its swimming towards targets. Mechanical stimuli near the head of the larva induce a fast escape response

(Granato *et al*., 1996; Saint-Amant and Drapeau, 1998). Tests like this on allow the identification of changes in sensory and locomotor functions (Stehr *et al*., 2006).

After the last reading of the third assay, each larva was displaced to a petri dish with autoclaved water. After a little period of habituation and stabilization (around 2 min), it was applied a short mechanical stimulus with a pipette tip in its head and tail. It was recorded the response of the larvae regarding escaping or don't (Table 5.1).

Regarding the responses from stimuli applied in the head, the differences obtained between the two controls (deionized water enriched with NaCl and deionized water with ascorbic acid enriched with NaCl) were not statistical significant ($p<0.05$). 33.3 % ($p<0.05$) of the larvae exposed to the BDM had a positive response (the larvae swam away from the stimulus). The larvae exposed to the DBPs showed 50 % ($p<0.05$) of positive response.

Regarding the responses from stimuli applied in the tail, 76.6 % of the larvae exposed to the BDM had a positive response (the larvae swam away from the stimulus) and the larvae exposed to the DBPs showed 66.6 % of positive response. However, these results don't have statistical significance too ($p>0.05$). The differences obtained between the two controls (deionized water enriched with NaCl and deionized water with ascorbic acid enriched with NaCl) were also not statistical significant ($p>0.05$) whereby it is not possible to conclude something about the results from the stimuli in the head.

Comparing the effects caused by BDM and the effects caused by the DBPs it was not observed significant differences ($p>0.05$) between the results at the end of the assay. So, no conclusions can be drawn from this touch response test.

Table 5.1. Percentage of positive reactions to the mechanic stimuli applied in the tail and the head of the larvae. Each experimental condition has two replications.

	Positive reaction (%)	
	tail	head
Autoclaved water	93,33	93,33
	100	93,33
NaCl Ctrl	86,67	80
	100	100
Ascorbic acid Ctrl	100	100
	93,33	93,33
0.1 mg/l BDM	86,67	33,33
	66,67	33,33
0.1 mg/L DBPs	60	40
	73,33	60

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Chapter VI

Conclusions

6. Conclusions

The present work had two major objectives: (i) the assess the stability of four UV-filters commonly used in PCPs: benzyl salicylate (BzS), phenyl salicylate (PS), 4-methyl benzilydene camphor (4-MBC) and octocrylene (OC) in chlorinated water, in conditions similar to those existent in swimming pools, (a) evaluating their stability in water samples with different pH values and different free chlorine conditions; (b) to determine their half-lives; (c) tentatively identify major DBPs and (d) to study their stability under time, and (ii) assess the toxicity of an UV-filter whose chlorination reaction already studied by Santos *et al*. (Santos *et al*., 2013) (BDM) comparing the toxicity caused by the parental compound (BDM) to the toxicity of its DBPs acutely exposing larvae of zebrafish (*Danio rerio*) to several concentrations of BDM and corresponding DBPs solution resulting from chlorination reaction. The effect of these compounds regarding mortality and anatomic abnormalities was assessed.

6.1. Study of the reaction kinetics of BzS, PS, 4-MCB and OC in chlorinated water

The kinetics of the reactions between the four UV-filters and chlorinated water, the DBPs formation and the effect of sample pH and free chlorine concentration were studied by HPLC-UV-DAD.

The studied salicylates (BzS and PS) react with chlorine leading to the formation of DBPs. PS was found to be less stable in chlorine and we think this reactivity difference is due to the possibility of a higher stabilization of the transition state of the electrophilic substitution reaction. Regarding 4-MBC and OC the chlorination reaction occurs but not in useful time.

DBPs of the two salicylates have been tentatively identified by HPLC-MS. Two mono- and one dichlorinated by-product have been detected for both salicylates. In contrast to what happens to the parental compounds, DBPs of BzS showed a good stability while PS by-products were found to significantly degrade after 20 min reaction. It was observed no DBPs for 4-MBC and OC.

To study the chlorination of the four UV-filters, free chlorine concentrations applied were from 1 mg/L and 10 mg/L. These concentrations are acceptable in several places around the world: free chlorine levels of less than 1 mg/L are acceptable in some countries but, in other countries higher levels are considered acceptable. In public and semi-public swimming pools and hot tubes it is acceptable levels of 3 mg/L and 5 mg/L of

free chlorine, respectively. However, periodically, in some situations there are applied concentrations up to 20 mg/L as a *shock dose* to better manage the microbial quality of the water (WHO, 2006). The pH should be maintained between 7.2 and 8.0 (WHO, 2006). So, in order to assess whether the two salicylates react to a significant extent with low chlorine concentrations, such as those used in swimming pools, a set of experiments was designed. The effect of three different pH (6.0, 7.0, 8.0) on the extent of the reaction between the two salicylates and four concentrations of free chlorine (1, 2, 3 and 5 mg/L) was studied separately for each salicylate. These experimental conditions were chosen with the intent to include the conditions used in swimming pool and hot tubs and this study was not applied to 4-MBC neither OC once these two UV-filters didn't react with chlorine in the previous one. It was possible to conclude that the extent of the reactions between BzS and PS and free chlorine depends on the studied parameters: concentration of chlorine and the pH of the water. It was observed that at pH 6.0 there is a small transformation of both salicylates (maximum about 20%) and is almost independently from free chlorine concentration. At pH 7.0 and 8.0 and for free chlorine concentrations from 2 to 5 mg/L the transformation percentage of BzS and PS increases with the increase of the solution pH. This can be due to the fact that the anionic forms of the salicylates (phenoxide anions) react faster with HOCl than the neutral forms with OCl^- [$pK_{HOCl,25ºC}$ = 7.54, (Deborde and Von Gunten, 2008)]. We can also conclude that at higher pH values PS reacts with chlorine in a higher extent than BzS.

6.2. Study of the degradation induced by UV radiation of 4-MBC and OC

Once the reaction with chlorine was not observed and to increase the knowledge about the reactions suffered by 4-MBC and OC in swimming pool water, the degradation induced by UV radiation was studied. It was verified that 4-MBC suffers isomerization between the *E*-form and the *Z*-form when under both artificial and natural UV radiation. This isomerization was already described by other authors (Giokas *et al.*, 2007; Rodil *et al*., 2009). The photostability of 4-MBC was already described (Gaspar and Maia Campos, 2006; Rodil *et al*., 2009) but here it was observed that, in presence of chlorine, the concentration of the *E*-form was lower than its concentration in absence of chlorine, while the concentration of the *Z*-form was similar with and without chlorine.

OC doesn't suffers isomerization and, although the photostability of OC was also already described (Giokas *et al.*, 2007; Rodil *et al*., 2009; (Kockler *et al.*, 2013), here it was observed the slightly decrease of the concentration of OC along the reaction time under natural UV radiation. The effect of chlorine was inconclusive since it was verified a

lower concentration of OC in presence of chlorine under artificial UV filter but the concentration under natural UV radiation was similar with and without chlorine.

6.3. Toxicological assays

Zebrafish is used as a toxicological model having several advantages for drug screening (McGrath and Li, 2009). This organism is small, cheap to keep in the laboratory and easily bred in large numbers. Larvae of zebrafish can live for seven days in a single well of microplates and their nutritional requirement is almost provided by nutrients stored in yolk sac. Zebrafish allows an easy administration of tested compounds because the larvae can absorb small molecules diluted in the medium through their skin and gills. Zebrafish has advantages comparing to other animal models since it is necessary small amounts of the tested drug, a large number of specimens can be used in each assay, and their organs and tissues (brain, heart, liver, pancreas, kidney, intestine, bones, muscles, nerve system and sensory organs) are similar to their mammalian counterparts regarding anatomical, physiological and molecular levels (McGrath and Li, 2009). The development of the zebrafish embryo is very fast. The larvae start feeding 5 dpf suggesting that most organs acquire a functional state at this time (Yang *et al.*, 2009).

Zebrafish was already utilized as animal model to assess the toxicity of OC by Blüthgen *et al.* (Blüthgen *et al.*, 2014) in a chronic exposure.

Here, it can be concluded that the presence of nutrients is essential for larvae survival and the concentration of NaCl used (5.03 mM) was enough to ensure the nutritional requirements. Ascorbic acid was a good way to eliminate free chlorine from the tested solutions and, although it was observed some skeletal deformations in the larvae exposed to the controls of ascorbic acid (fourth assay), these results were not statistically significant.

It was not possible to conclude if BDM and its DBPs at 1 mg/L have impact on the larvae (second assay) although it was observed some abnormalities at 0.1 mg/L of DBPs (third assay). On the other hand, BDM at lower concentrations (0.1 µg/L) (fourth assay) caused mortality as well as DBPs at 0.1 µg/L, 1 µg/L and 10 µg/L. Usually, UV-filters have a high lipophilicity (Díaz-Cruz and Barceló, 2009) and it was observed that, after the end of the assays, the wells containing the BDM solutions showed an oily residue what may indicate a poor solubility of BDM in the water. So, this poor solubility may explain the high mortality caused at lower concentrations of BDM since at the higher concentration BDM could not be completely dissolved forming film which is not contacted with the larvae.

Regarding the touch response test, it was not observed significant differences between the effects caused by BDM and the effects caused by the DBPs at the end of the assay. Generally, the percentage of positive reactions of the stimuli applied in the tail was higher than the percentage of positive reactions of the stimuli applied in the head (Table 5.1) During the development of the larvae, the movements of the tail appears first than the movements of the head (Saint-Amant and Drapeau, 1998) so, it is normal that the experimental conditions affect the escape response induced by a stimulus near the head more than near the tail. It was also observed that all the larvae with some abnormality have negative responses to these stimuli.

The concentrations used in these assays viewed to coincide with the levels of UV-filters usually found in the environmental (Zhang and Lee, 2013). After an optimization process it was possible test concentrations raging µg/L. However, due to the method used to obtain the DBPs, it was not possible ensure that the real concentration of DBPs present in the solutions was the calculated. The fact that the concentration decreases along the time is another reason to not be possible to ensure that the concentrations were the expected. Now, it is important to develop methods to optimize the determination of the real concentrations.

In the natural ecosystems, the organisms are exposed to mixtures of many compounds whereby the effects observed may be synergistic even when the individual compounds are present at no observable effect concentrations (NOECs) (Díaz-Cruz and Barceló, 2009). As sunscreens are applied containing several UV-filters, it is critical to assess their activity in mixture combinations (Díaz-Cruz and Barceló, 2009).

Future work should investigate the presence of these DBPs in swimming pool water and waste water, address potential risks for human health due to dermal contact and evaluate possible environmental toxic effects. Environmental chemistry studies should also focus on strategies to minimize the formation of these DBPs by the development of new formulations that prevent PPCPs release into chlorinated water and strategies to reduce PPCPs by pool water treatment and by pre-swim showering.

From the point of view of the water quality of swimming pools where the PPCPs used by swimmers are released to water this work is quite relevant because these PPCPs and their transformation products accumulate in the bathing water rising human health concerns. Environmental risk can also arise after discharge of the swimming pool effluents into the aquatic systems.

6.4. References

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Appendix

Appendix A.1. Percentage of mortality (left column) and abnormalities (right column) in zebrafish caused by different conditions per well along all the days of the second assay and corresponding results from statistical analysis (One-way ANOVA).

			Mortality (%)			Abnormalinities (%)	
			BDM	BDPs		BDM	BDPs
2º day	BDM/BDPs	1	0	0		0	0
		2	0	0		0	0
		3	0	20		0	0
			BDPs	0.30 ppm Cl Ctrl		BDPs	0.30 ppm Cl Ctrl
	BDPs/0.30 ppm Cl Ctrl	1	0	0		0	0
		2	0	0		0	0
		3	20	0		0	0
			BDM	BDPs		BDM	BDPs
3º day	BDM/BDPs	1	0	0		0	60
		2	0	0		0	0
		3	0	0		0	0
			BDPs	0.30 ppm Cl Ctrl		BDPs	0.30 ppm Cl Ctrl
	BDPs/0.30 ppm Cl Ctrl	1	0	0		60	20
		2	0	0		0	20
		3	20	0		0	0
			BDM	BDPs		BDM	BDPs
4º day	BDM/BDPs	1	0	0		0	40
		2	0	0		0	0
		3	0	0		0	0
			BDPs	0.30 ppm Cl Ctrl		BDPs	0.30 ppm Cl Ctrl
	BDPs/0.30 ppm Cl Ctrl	1	0	0		40	20
		2	0	0		0	0
		3	20	0		0	0

Mortality

2º day — BDM/BDPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666667	133,3333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66667			
Total	333,3333	5				

2º day — BDPs/0.30 ppm Cl Ctrl.

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	20	6,666667	133,3333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66667			
Total	333,3333	5				

Abnormalities

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

3º day

BDM/BDPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	60	20	1200

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	600	1	600	1	0,373901	7,708647
Within Groups	2400	4	600			
Total	3000	5				

BDPs/0.30 ppm Cl Ctrl.

Anova: factor único

SUMÁRIO

Grupos	Contagem	Soma	Méday	Variância
Coluna 1	3	20	6,666667	133,3333
Coluna 2	3	0	0	0

ANOVA

Fonte de variação	SQ	gl	MQ	F	valor P	F crítico
Entre grupos	66,66667	1	66,66667	1	0,373901	7,708647
Dentro de grupos	266,6667	4	66,66667			
Total	333,3333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	60	20	1200
Column 2	3	40	13,33333	133,3333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66667	0,1	0,767644	7,708647
Within Groups	2666,667	4	666,6667			
Total	2733,333	5				

4º day

BDM/BDPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	40	13,33333	533,3333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	266,6667	1	266,6667	1	0,373901	7,708647
Within Groups	1066,667	4	266,6667			
Total	1333,333	5				

BDPs/0.30 ppm Cl Ctrl.

Anova: factor único

SUMÁRIO

Grupos	Contagem	Soma	Méday	Variância
Coluna 1	3	20	6,666667	133,3333
Coluna 2	3	0	0	0

ANOVA

Fonte de variação	SQ	gl	MQ	F	valor P	F crítico
Entre grupos	66,66667	1	66,66667	1	0,373901	7,708647
Dentro de grupos	266,6667	4	66,66667			
Total	333,3333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	40	13,33333	533,3333
Column 2	3	20	6,666667	133,3333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66667	0,2	0,677869	7,708647
Within Groups	1333,333	4	333,3333			
Total	1400	5				

2º day

Ctrl Cl 0.3 ppm/0.30 ppm Cl + NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

NaCl Ctrl/Autoclaved water

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

3º day

Ctrl Cl 0.3 ppm/0.30 ppm Cl + NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	100	33,33333	933,3333
Column 2	3	40	13,33333	133,3333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	600	1	600	1,125	0,348641	7,708647
Within Groups	2133,333	4	533,3333			
Total	2733,333	5				

NaCl Ctrl/Autoclaved water

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

4º day

Ctrl Cl 0.3 ppm/0.30 ppm Cl + NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666667	133,3333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66667			
Total	333,3333	5				

NaCl Ctrl/Autoclaved water

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666667	133,3333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66667			
Total	333,3333	5				

Appendix A.2. Percentage of mortality (left column) and abnormalities (right column) in zebrafish caused by different conditions per well along all the days of the third assay and corresponding results from statistical analysis (One-way ANOVA).

			Mortality (%)			Abnormalities (%)	
			BDM	DBPs		BDM	DBPs
2º day	BDM/DBPs	1	0	0		0	0
		2	0	0		20	6,67
	Autoclaved water		w/o Ascorbic Acid	w/ Ascorbic Acid		w/o Ascorbic Acid	w/ Ascorbic Acid
		1	0	0		0	0
		2	0	0		0	0
			BDM	DBPs		BDM	DBPs
3º day	BDM/DBPs	1	0	0		0	0
		2	0	0		6,67	0
	H2O		w/o Ascorbic Acid	w/ Ascorbic Acid		w/o Ascorbic Acid	w/ Ascorbic Acid
		1	0	0		6,67	0
		2	0	0		0	0
			BDM	DBPs		BDM	DBPs
4º day	BDM/DBPs	1	0	0		60	0
		2	0	0		60	0
	H2O		w/o Ascorbic Acid	w/ Ascorbic Acid		w/o Ascorbic Acid	w/ Ascorbic Acid
		1	0	0		6,67	0
		2	0	0		0	0
			BDM	DBPs		BDM	DBPs
5º day	BDM/DBPs	1	0	0		13,33	0
		2	0	0		13,33	0
	H2O		w/o Ascorbic Acid	w/ Ascorbic Acid		w/o Ascorbic Acid	w/ Ascorbic Acid
		1	0	0		6,67	0
		2	0	0		0	0

2º day

BDM/DBPs

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2	20	10	200
Column 2	2	6,67	3,335	22,24445

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	44,42223	1	44,42223	0,39976	0,591854	18,51282
Within Groups	222,2445	2	111,1222			
Total	266,6667	3				

2º day

Ascorbic Acid

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

3º day

BDM/DBPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	6,67	3,335	22,24445
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	11,12223	1	11,12223	1	0,42265	18,51282
Within Groups	22,24445	2	11,12223			
Total	33,36668	3				

Ascorbic Acid

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	6,67	3,335	22,24445
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	11,12223	1	11,12223	1	0,42265	18,51282
Within Groups	22,24445	2	11,12223			
Total	33,36668	3				

4º day

BDM/DBPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	120	60	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3600	1	3600	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	3600	3				

Ascorbic Acid

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	6,67	3,335	22,24445
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	11,12223	1	11,12223	1	0,42265	18,51282
Within Groups	22,24445	2	11,12223			
Total	33,36668	3				

5º day

BDM/DBPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	26,66	13,33	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	177,6889	1	177,6889	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	177,6889	3				

Ascorbic Acid

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	6,67	3,335	22,24445
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	11,12223	1	11,12223	1	0,42265	18,51282
Within Groups	22,24445	2	11,12223			
Total	33,36668	3				

		Mortality (%)			Abnormalities (%)		
		BDM	NaCl Ctrl		BDM	NaCl Ctrl	
2º day	BDM/NaCl Ctrl	1	0	0	0	0	
		2	0	0	20	0	
	DBPs/Ascorbic acid + NaCl Ctrl	DBPs	Ascorbic acid + NaCl Ctrl		DBPs	Ascorbic acid + NaCl Ctrl	
		1	0	0	6,67	0	
		2	0	0	0	0	
		BDM	NaCl Ctrl		BDM	NaCl Ctrl	
3º day	BDM/NaCl Ctrl	1	0	0	0	6,67	
		2	0	0	6,67	0	
	DBPs/Ascorbic acid + NaCl Ctrl	DBPs	Ascorbic acid + NaCl Ctrl		DBPs	Ascorbic acid + NaCl Ctrl	
		1	0	0	0	0	
		2	0	0	0	0	
		BDM	NaCl Ctrl		BDM	NaCl Ctrl	
4º day	BDM/NaCl Ctrl	1	0	0	60	6,67	
		2	0	0	60	0	
	DBPs/Ascorbic acid + NaCl Ctrl	DBPs	Ascorbic acid + NaCl Ctrl		DBPs	Ascorbic acid + NaCl Ctrl	
		1	0	0	0	0	
		2	0	0	0	0	
		BDM	NaCl Ctrl		BDM	NaCl Ctrl	
5º day	BDM/NaCl Ctrl	1	0	0	13,33	6,67	
		2	0	0	13,33	0	
	DBPs/Ascorbic acid + NaCl Ctrl	DBPs	Ascorbic acid + NaCl Ctrl		DBPs	Ascorbic acid + NaCl Ctrl	
		1	0	0	0	0	
		2	0	0	0	0	

2º day

BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	20	10	200
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	100	1	100	1	0,42265	18,51282
Within Groups	200	2	100			
Total	300	3				

DBPs/Ascorbic acid + NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	6,67	3,335	22,24445
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	11,12223	1	11,12223	1	0,42265	18,51282
Within Groups	22,24445	2	11,12223			
Total	33,36668	3				

3º day

BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	6,67	3,335	22,24445
Column 2	2	6,67	3,335	22,24445

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	0	1	18,51282
Within Groups	44,4889	2	22,24445			
Total	44,4889	3				

DBPs/Ascorbic acid + NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

4º day

BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	120	60	0
Column 2	2	6,67	3,335	22,24445

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3210,922	1	3210,922	288,6942	0,003446	18,51282
Within Groups	22,24445	2	11,12223			
Total	3233,167	3				

DBPs/Ascorbic acid + NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

5º day

BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	26,66	13,33	0
Column 2	2	6,67	3,335	22,24445

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	99,90003	1	99,90003	8,982018	0,095631	18,51282
Within Groups	22,24445	2	11,12223			
Total	122,1445	3				

DBPs/Ascorbic acid + NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	2	0	0	0
Column 2	2	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	18,51282
Within Groups	0	2	0			
Total	0	3				

Appendix A.3. Percentage of mortality (left column) and abnormalities (right column) in zebrafish caused by different conditions per well along all the days of the fourth assay and corresponding results from statistical analysis (One-way ANOVA).

			Mortality (%)			Abnormalities (%)		
			10 ug/L	1 ug/L	0.1 ug/L	10 ug/L	1 ug/L	0.1 ug/L
2º day	BDM	1	0	0	0	0	0	20
		2	0	0	20	0	20	0
		3	0	0	20	0	20	20
	DBPs	1	100	80	0	0	0	20
		2	100	100	0	0	0	60
		3	100	60	0	0	0	20
			w/o Ascorbic acid	w/ Ascorbic acid		w/o Ascorbic acid	w/ Ascorbic acid	
	Autoclaved water	1	0	60		0	20	
		2	0	40		0	0	
		3	0	0		0	20	
			10 ug/L	1 ug/L	0.1 ug/L	10 ug/L	1 ug/L	0.1 ug/L
3º day	BDM	1	0	0	100	0	0	0
		2	0	0	100	0	0	0
		3	0	0	80	0	0	0
	DBPs	1	100	80	0	0	0	20
		2	100	100	0	0	0	60
		3	100	60	0	0	0	20
			w/o Ascorbic acid	w/ Ascorbic acid		w/o Ascorbic acid	w/ Ascorbic acid	
	Autoclaved water	1	0	60		0	20	
		2	0	40		0	0	
		3	0	0		0	20	
			10 ug/L	1 ug/L	0.1 ug/L	10 ug/L	1 ug/L	0.1 ug/L
4º day	BDM	1	0	0	100	20	0	0
		2	0	0	100	0	0	0
		3	0	0	80	0	0	0
	DBPs	1	100	80	20	0	0	20
		2	100	100	60	0	0	60
		3	100	60	20	0	0	20
			w/o Ascorbic acid	w/ Ascorbic acid		w/o Ascorbic acid	w/ Ascorbic acid	
	Autoclaved water	1	0	80		0	0	
		2	0	60		0	0	
		3	0	20		20	0	
			10 ug/L	1 ug/L	0.1 ug/L	10 ug/L	1 ug/L	0.1 ug/L
5º day	BDM	1	0	0	100	20	0	0
		2	0	0	100	0	0	0
		3	0	0	80	0	0	20
	DBPs	1	100	100	100	0	0	0
		2	100	100	100	0	0	0
		3	100	100	100	0	0	0
			w/o Ascorbic acid	w/ Ascorbic acid		w/o Ascorbic acid	w/ Ascorbic acid	
	Autoclaved water	1	0	0		80	0	
		2	0	0		100	0	
		3	0	0		20	0	

2º day

BDM

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0
Column 3	3	40	13,33333333	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	355,5555556	2	177,7777778	4	0,078717201	5,14325285
Within Groups	266,6666667	6	44,44444444			
Total	622,2222222	8				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	40	13,33333333	133,3333333
Column 3	3	40	13,33333333	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	355,5555556	2	177,7777778	2	0,216	5,14325285
Within Groups	533,3333333	6	88,88888889			
Total	888,8888889	8				

DBPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	240	80	400
Column 3	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	16800	2	8400	63	9,39144E-05	5,14325285
Within Groups	800	6	133,3333333			
Total	17600	8				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0
Column 3	3	100	33,33333333	533,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2222,222222	2	1111,111111	6,25	0,03411	5,14325285
Within Groups	1066,666667	6	177,7777778			
Total	3288,888889	8				

Ascorbic acid Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	100	33,33333333	933,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1666,666667	1	1666,666667	3,571428571	0,131777567	7,70864742
Within Groups	1866,666667	4	466,6666667			
Total	3533,333333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	40	13,33333333	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	266,6666667	1	266,6666667	4	0,11612	7,70864742
Within Groups	266,6666667	4	66,66666667			
Total	533,3333333	5				

3º day

BDM

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0
Column 3	3	280	93,33333333	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	17422,22222	2	8711,111111	196	3,42614E-06	5,14325285
Within Groups	266,6666667	6	44,44444444			
Total	17688,88889	8				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0
Column 3	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	2	0	65535	#DIV/0!	5,14325285
Within Groups	0	6	0			
Total	0	8				

DBPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	240	80	400
Column 3	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	16800	2	8400	63	9,39144E-05	5,14325285
Within Groups	800	6	133,3333333			
Total	17600	8				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0
Column 3	3	100	33,33333333	533,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2222,222222	2	1111,111111	6,25	0,03411	5,14325285
Within Groups	1066,666667	6	177,7777778			
Total	3288,888889	8				

Ascorbic acid Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	100	33,33333333	933,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1666,666667	1	1666,666667	3,571428571	0,131777567	7,70864742
Within Groups	1866,666667	4	466,6666667			
Total	3533,333333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	40	13,33333333	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	266,6666667	1	266,6666667	4	0,11612	7,70864742
Within Groups	266,6666667	4	66,66666667			
Total	533,3333333	5				

4º day

BDM

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0
Column 3	3	280	93,33333333	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	17422,22222	2	8711,111111	196	3,42614E-06	5,14325285
Within Groups	266,6666667	6	44,44444444			
Total	17688,88889	8				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	20	6,666666667	133,3333333
Column 2	3	0	0	0
Column 3	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	88,88888889	2	44,44444444	1	0,42187	5,14325285
Within Groups	266,6666667	6	44,44444444			
Total	355,5555556	8				

DBPs

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	240	80	400
Column 3	3	100	33,33333333	533,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	7022,222222	2	3511,111111	11,28571429	0,009261	5,14325285
Within Groups	1866,666667	6	311,1111111			
Total	8888,888889	8				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0
Column 3	3	100	33,33333333	533,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2222,222222	2	1111,111111	6,25	0,03411	5,14325285
Within Groups	1066,666667	6	177,7777778			
Total	3288,888889	8				

Ascorbic acid Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	160	53,33333333	933,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4266,666667	1	4266,666667	9,142857143	0,039020527	7,70864742
Within Groups	1866,666667	4	466,6666667			
Total	6133,333333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	20	6,666666667	133,3333333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66666667	1	66,66666667	1	0,3739	7,70864742
Within Groups	266,6666667	4	66,66666667			
Total	333,3333333	5				

5º day

BDM

Anova: factor único

SUMÁRIO

Grupos	Contagem	Soma	Méday	Variância
Coluna 1	3	0	0	0
Coluna 2	3	0	0	0
Coluna 3	3	280	93,33333333	133,3333333

ANOVA

Fonte de variação	SQ	gl	MQ	F	valor P	F crítico
Entre grupos	17422,22222	2	8711,111111	196	3,42614E-06	5,14325285
Dentro de grupos	266,6666667	6	44,44444444			
Total	17688,88889	8				

Anova: factor único

SUMÁRIO

Grupos	Contagem	Soma	Méday	Variância
Coluna 1	3	20	6,666666667	133,3333333
Coluna 2	3	0	0	0
Coluna 3	3	20	6,666666667	133,3333333

ANOVA

Fonte de variação	SQ	gl	MQ	F	valor P	F crítico
Entre grupos	88,88888889	2	44,44444444	0,5	0,62974	5,14325285
Dentro de grupos	533,3333333	6	88,88888889			
Total	622,2222222	8				

DBPs

Anova: factor único

SUMÁRIO

Grupos	Contagem	Soma	Méday	Variância
Coluna 1	3	300	100	0
Coluna 2	3	300	100	0
Coluna 3	3	300	100	0

ANOVA

Fonte de variação	SQ	gl	MQ	F	valor P	F crítico
Entre grupos	0	2	0	65535	#DIV/0!	5,14325285
Dentro de grupos	0	6	0			
Total	0	8				

Anova: factor único

SUMÁRIO

Grupos	Contagem	Soma	Méday	Variância
Coluna 1	3	20	6,666666667	133,3333333
Coluna 2	3	0	0	0
Coluna 3	3	20	6,666666667	133,3333333

ANOVA

Fonte de variação	SQ	gl	MQ	F	valor P	F crítico
Entre grupos	88,88888889	2	44,44444444	0,5	0,62974	5,14325285
Dentro de grupos	533,3333333	6	88,88888889			
Total	622,2222222	8				

Ascorbic acid Ctrl

Anova: factor único

SUMÁRIO

Grupos	Contagem	Soma	Méday	Variância
Coluna 1	3	0	0	0
Coluna 2	3	0	0	0

ANOVA

Fonte de variação	SQ	gl	MQ	F	valor P	F crítico
Entre grupos	0	1	0	65535	#DIV/0!	7,70864742
Dentro de grupos	0	4	0			
Total	0	5				

Anova: factor único

SUMÁRIO

Grupos	Contagem	Soma	Méday	Variância
Coluna 1	3	200	66,66666667	1733,333333
Coluna 2	3	0	0	0

ANOVA

Fonte de variação	SQ	gl	MQ	F	valor P	F crítico
Entre grupos	6666,666667	1	6666,666667	7,692307692	0,05015	7,70864742
Dentro de grupos	3466,666667	4	866,6666667			
Total	10133,33333	5				

			Mortality (%)			Abnormalities (%)	
			BDM	H2O NaCl		BDM	H2O NaCl
2º day	0,1 mg/L BDM/H2O NaCl	1	0	0		20	0
		2	20	0		0	0
		3	20	0		20	0
	1 mg/L BDM/H2O NaCl	1	0	0		0	0
		2	0	0		20	0
		3	0	0		20	0
			BDM	H2O NaCl		BDM	H2O NaCl
	10 mg/L BDM/H2O NaCl	1	0	0		0	0
		2	0	0		0	0
		3	0	0		0	0
			BDM	H2O NaCl		BDM	H2O NaCl
3º day	0,1 mg/L BDM/H2O NaCl	1	100	0		0	0
		2	100	0		0	0
		3	80	0		0	20
	1 mg/L BDM/H2O NaCl	1	0	0		0	0
		2	0	0		0	0
		3	0	0		0	20
			BDM	H2O NaCl		BDM	H2O NaCl
	10 mg/L BDM/H2O NaCl	1	0	0		0	0
		2	0	0		0	0
		3	0	0		0	20
			BDM	H2O NaCl		BDM	H2O NaCl
4º day	0,1 mg/L BDM/H2O NaCl	1	100	0		0	0
		2	100	0		0	0
		3	80	0		0	20
	1 mg/L BDM/H2O NaCl	1	0	0		0	0
		2	0	0		0	0
		3	0	0		0	20
			BDM	H2O NaCl		BDM	H2O NaCl
	10 mg/L BDM/H2O NaCl	1	0	0		20	0
		2	0	0		0	0
		3	0	0		0	20
			BDM	H2O NaCl		BDM	H2O NaCl
5º day	0,1 mg/L BDM/H2O NaCl	1	100	0		0	0
		2	100	0		0	0
		3	80	0		20	0
	1 mg/L BDM/H2O NaCl	1	0	0		0	0
		2	0	0		0	0
		3	0	0		0	0
			BDM	H2O NaCl		BDM	H2O NaCl
	10 mg/L BDM/H2O NaCl	1	0	0		20	0
		2	0	0		0	0
		3	0	0		0	0

2º day

0,1 mg/L BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	40	13,33333	133,3333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	266,6667	1	266,6667	4	0,116116524	7,708647
Within Groups	266,6667	4	66,66667			
Total	533,3333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	40	13,33333333	133,3333333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	266,6667	1	266,6666667	4	0,116117	7,708647
Within Groups	266,6667	4	66,66666667			
Total	533,3333	5				

1 mg/L BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	40	13,33333333	133,3333333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	266,6667	1	266,6666667	4	0,116117	7,708647
Within Groups	266,6667	4	66,66666667			
Total	533,3333	5				

10 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

3º day

0,1 mg/L BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	280	93,33333	133,3333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	13066,67	1	13066,67	196	0,000151011	7,708647
Within Groups	266,6667	4	66,66667			
Total	13333,33	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

1 mg/L BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

10 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

4º day

0,1 mg/L BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	280	93,33333	133,3333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	13066,67	1	13066,67	196	0,000151011	7,708647
Within Groups	266,6667	4	66,66667			
Total	13333,33	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

1 mg/L BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

10 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	20	6,666666667	133,3333333
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	0	1	7,708647
Within Groups	533,3333	4	133,3333333			
Total	533,3333	5				

5º day

0,1 mg/L BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	280	93,33333	133,3333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	13066,67	1	13066,67	196	0,000151011	7,708647
Within Groups	266,6667	4	66,66667			
Total	13333,33	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	20	6,666666667	133,3333333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

1 mg/L BDM/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

10 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	20	6,666666667	133,3333333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

			Mortality (%)			Abnormalities (%)	
		DBPs	H2O NaCl		DBPs	H2O NaCl	
2º day	0,1 mg/L DBPs/NaCl Ctrl	1	0	0		20	0
		2	0	0		60	0
		3	0	0		20	0
	1 mg/L DBPs/NaCl Ctrl	1	80	0		0	0
		2	100	0		0	0
		3	60	0		0	0
		DBPs	H2O NaCl		DBPs	H2O NaCl	
	10 mg/L DBPs/NaCl Ctrl	1	100	0		0	0
		2	100	0		0	0
		3	100	0		0	0
		DBPs	NaCl Ctrl		DBPs	NaCl Ctrl	
3º day	0,1 mg/L DBPs/NaCl Ctrl	1	20	0		0	0
		2	60	0		60	0
		3	20	0		20	20
	1 mg/L DBPs/NaCl Ctrl	1	80	0		0	0
		2	100	0		0	0
		3	60	0		0	20
		DBPs	NaCl Ctrl		DBPs	NaCl Ctrl	
	10 mg/L DBPs/NaCl Ctrl	1	100	0		0	0
		2	100	0		0	0
		3	100	0		0	20
		DBPs	NaCl Ctrl		DBPs	NaCl Ctrl	
4º day	0,1 mg/L DBPsNaCl Ctrl	1	20	0		20	0
		2	60	0		60	0
		3	20	0		20	20
	1 mg/L DBPs/NaCl Ctrl	1	80	0		0	0
		2	100	0		0	0
		3	60	0		0	20
		DBPs	NaCl Ctrl		DBPs	NaCl Ctrl	
	10 mg/L DBPs/NaCl Ctrl	1	100	0		0	0
		2	100	0		0	0
		3	100	0		0	20
		DBPs	NaCl Ctrl		DBPs	NaCl Ctrl	
5º day	0,1 mg/L DBPs/NaCl Ctrl	1	100	0		0	0
		2	100	0		0	0
		3	100	0		0	0
	1 mg/L DBPs/NaCl Ctrl	1	100	0		0	0
		2	100	0		0	0
		3	100	0		0	0
		DBPs	NaCl Ctrl		DBPs	NaCl Ctrl	
	10 mg/L DBPs/NaCl Ctrl	1	100	0		0	0
		2	100	0		0	0
		3	100	0		0	0

2º day

0,1 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	100	33,33333	533,3333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1666,667	1	1666,667	6,25	0,066766545	7,708647
Within Groups	1066,667	4	266,6667			
Total	2733,333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	100	33,33333333	533,3333333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1666,667	1	1666,666667	6,25	0,066767	7,708647
Within Groups	1066,667	4	266,6666667			
Total	2733,333	5				

1 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	240	80	400
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9600	1	9600	48	0,002278426	7,708647
Within Groups	800	4	200			
Total	10400	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

10 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	15000	1	15000	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	15000	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

3º day

0,1 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	100	33,33333	533,3333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1666,667	1	1666,667	6,25	0,066766545	7,708647
Within Groups	1066,667	4	266,6667			
Total	2733,333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	80	26,66666667	933,3333333
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	600	1	600	1,125	0,348641	7,708647
Within Groups	2133,333	4	533,3333333			
Total	2733,333	5				

1 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	240	80	400
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9600	1	9600	48	0,002278426	7,708647
Within Groups	800	4	200			
Total	10400	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

10 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	15000	1	15000	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	15000	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

4º day

0,1 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	100	33,33333	533,3333
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1666,667	1	1666,667	6,25	0,066766545	7,708647
Within Groups	1066,667	4	266,6667			
Total	2733,333	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	100	33,33333333	533,3333333
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1066,667	1	1066,666667	3,2	0,148148	7,708647
Within Groups	1333,333	4	333,3333333			
Total	2400	5				

1 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	240	80	400
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9600	1	9600	48	0,002278426	7,708647
Within Groups	800	4	200			
Total	10400	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

10 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	15000	1	15000	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	15000	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	20	6,666666667	133,3333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66,66667	1	66,66666667	1	0,373901	7,708647
Within Groups	266,6667	4	66,66666667			
Total	333,3333	5				

5º day

0,1 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	15000	1	15000	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	15000	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

1 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	15000	1	15000	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	15000	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

10 mg/L DBPs/NaCl Ctrl

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	300	100	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	15000	1	15000	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	15000	5				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	3	0	0	0
Column 2	3	0	0	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0	1	0	65535	#DIV/0!	7,708647
Within Groups	0	4	0			
Total	0	5				

Appendix A.4. Percentage of positive responses in tail (left column) and in head (right column) in zebrafish durinh the touch response test in zebrafish exposed to different conditions and corresponding results from statistical analysis (One-way ANOVA).

Toutch Response Test

		tail		head	
	BDM	DBPs	BDM	DBPs	
BDM/DBPs	1	86,67	60	33,33	40
	2	66,67	73,33	33,33	60
		w/ Ascorbic Acid	w/o Ascorbic Acid	w/ Ascorbic acid	w/o Ascorbic Acid
Autoclaved water	1	86,67	100	80	33,33
	2	100	93,33	100	33,33

Tail

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2	153,34	76,67	200
Column 2	2	133,33	66,665	88,84445

BDM/DBPs

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	100,100025	1	100,100025	0,693106792	0,49269	18,51282051
Within Groups	288,84445	2	144,42225			
Total	388,944475	3				

Head

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2	66,66	33,33	0
Column 2	2	100	50	200

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	277,8889	1	277,8889	2,778889	0,2374443323	18,51282051
Within Groups	200	2	100			
Total	477,8889	3				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2	186,67	93,335	88,84445
Column 2	2	193,33	96,665	22,24445

Ascorbic Acid

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11,0889	1	11,0889	0,199640108	0,698735	18,51282051
Within Groups	111,0889	2	55,54445			
Total	122,1778	3				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2	180	90	200
Column 2	2	66,66	33,33	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3211,489	1	3211,489	32,11489	0,029755407	18,51282051
Within Groups	200	2	100			
Total	3411,4889	3				

		tail		head	
	BDM	H2O NaCl	BDM	H2O NaCl	
BDM/NaCl Ctrl	1	86,67	86,67	33,33	80
	2	66,67	100	33,33	100

		DBPs	Ascorbic acid + NaCl	DBPs	Ascorbic acid + NaCl Ctrl
		Ascorbic acid + NaCl Ctrl			
DBPs/Ascorbic acid + NaCl Ctrl	1	60	100	80	33,33
	2	73,33	93,33	100	33,33

	Tail						
BDM/NaCl Ctrl	Anova: Single Factor						
	SUMMARY						
	Groups	*Count*	*Sum*	*Average*	*Variance*		
	Column 1	2	153,34	76,67	200		
	Column 2	2	186,67	93,335	88,84445		
	ANOVA						
	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Between Groups	277,722225	1	277,722225	1,922988134	0,299868	18,51282051
	Within Groups	288,84445	2	144,422225			
	Total	566,566675	3				
DBPs/Ascorbic acid + NaCl Ctrl	Anova: Single Factor						
	SUMMARY						
	Groups	*Count*	*Sum*	*Average*	*Variance*		
	Column 1	2	133,33	66,665	88,84445		
	Column 2	2	193,33	96,665	22,24445		
	ANOVA						
	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Between Groups	900	1	900	16,20323903	0,056533	18,51282051
	Within Groups	111,0889	2	55,54445			
	Total	1011,0889	3				

	Head						
	Anova: Single Factor						
	SUMMARY						
	Groups	*Count*	*Sum*	*Average*	*Variance*		
	Column 1	2	66,66	33,33	0		
	Column 2	2	180	90	200		
	ANOVA						
	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Between Groups	3211,4889	1	3211,489	32,11489	0,029755407	18,51282051
	Within Groups	200	2	100			
	Total	3411,4889	3				
	Anova: Single Factor						
	SUMMARY						
	Groups	*Count*	*Sum*	*Average*	*Variance*		
	Column 1	2	180	90	200		
	Column 2	2	66,66	33,33	0		
	ANOVA						
	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Between Groups	3211,4889	1	3211,489	32,11489	0,029755407	18,51282051
	Within Groups	200	2	100			
	Total	3411,4889	3				