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Ecotoxicity of oxidative stress effectors

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Resumo

O stress oxidativo ocorre quando existe um desequilíbrio na regulação de reações redox num sistema biológico a favor dos oxidantes. A interação de radicais livres, espécies reativas que atuam como pró-oxidantes, com várias biomoléculas é responsável por alterações que estão envolvidas na patogénese de várias doenças. Quando essa interação ocorre com lípidos, designa-se por peroxidação lipídica. O principal alvo da peroxidação lipídica são os ácidos gordos polinsaturados e, quando oxidados, geram de forma não-enzimática biomoléculas denominadas isoprostanos. Assim, os isoprostanos são considerados produtos finais duma peroxidação lipídica e, por isso, possíveis indicadores da existência dum estado de stress oxidativo associado a várias patologias.

Tendo em conta que os isoprostanos são excretados através da urina, estes podem ser detetados em efluentes de estações de tratamento de águas residuais e, por isso, ser usados como indicadores na avaliação do estado de saúde duma população. Contudo, o facto dos isoprostanos estarem comprovadamente presentes em efluentes pode levantar preocupações sobre a integridade do ambiente aquático, que no presente se restringem à esfera do hipotético, uma vez que a sua potencial toxicidade para espécies aquáticas permanece por explorar. Assim, sugere-se a hipótese de que os isoprostanos possam iniciar um fenómeno de toxicidade circular: o ser humano, quando num estado de stress oxidativo, excreta isoprostanos através da urina sendo estes encaminhados para estações de tratamento de águas residuais que não possuem capacidade para a sua remoção. Desta forma, os isoprostanos entram nos ecossistemas aquáticos onde podem interagir com as múltiplas espécies possuidoras de recetores que os reconhecem.

Para testar esta hipótese, foram realizados ensaios de toxicidade aguda e crónica com o organismo modelo *Daphnia magna* por forma a avaliar a toxicidade de efetores de stress oxidativo (hipoclorito de sódio e isoprostanos). Para além disso, realizaram-se também testes multigeracionais e transgeracionais com o objetivo de perceber se estes compostos induzem efeitos em gerações que não foram cronicamente expostas.

Tendo em conta os resultados obtidos, foi possível concluir que a exposição crónica de dáfnias quer a hipoclorito de sódio, quer a isoprostanos, leva a que o sucesso reprodutivo seja dependente da concentração dos compostos. No entanto, apenas o hipoclorito de sódio afetou o tamanho das dáfnias, indiciando um mecanismo de atuação diferente para os dois compostos. Para além disso, foi também possível concluir que ambos os compostos são capazes de induzir efeitos multigeracionais e transgeracionais relativamente ao sucesso reprodutivo. Nestas circunstâncias, foi possível validar a hipótese inicial da existência de toxicidade circular associada aos isoprostanos.

Palavras-chave

Stress oxidativo, Peroxidação lipídica, Isoprostanos, *Daphnia magna*, Toxicidade circular.

Resumo alargado

Num sistema biológico o estado de homeostasia nas reações de oxirredução pode ser alterado por meio de desafios redox. Estes determinam a expressão de genes que irão contrariar efeitos potencialmente deletérios. Ao nível fisiológico, as espécies antioxidantes e pró-oxidantes encontram-se em equilíbrio, permitindo simultaneamente a sinalização e a regulação dos níveis redox. No entanto, certos *stressors* podem desencadear um nível de resposta conducente ao desequilíbrio deste estado homeostático no sentido do aumento de oxidantes, levando à prevalência de um estado de stress oxidativo suprafisiológico que interrompe a sinalização e/ou danifica biomoléculas. A interação de radicais livres com várias biomoléculas é responsável por alterações que estão envolvidas na patogénese de várias doenças e, no caso específico dos lípidos, designa-se por peroxidação lipídica.

O principal alvo da peroxidação lipídica são os ácidos gordos polinsaturados, uma classe de lípidos mais reativos devido à sua estrutura, que quando oxidados, geram de forma não-enzimática biomoléculas denominadas isoprostanos. Assim sendo, os isoprostanos são produtos finais de um processo de peroxidação lipídica, por consequência desencadeada pela ação de radicais livres, podendo indicar a existência de um estado de stress oxidativo.

Assim, a deteção de níveis elevados de isoprostanos corresponderá, por norma, a níveis elevados de stress oxidativo, podendo ser utilizados como indicadores de certas patologias. Tendo em conta que os isoprostanos são excretados através da urina, estes podem ser detetados em efluentes de estações de tratamento de águas residuais e, por isso, ser usados como indicadores na avaliação do estado de saúde duma população.

Contudo, o facto dos isoprostanos estarem comprovadamente presentes em efluentes pode levantar preocupações sobre a integridade do ambiente aquático, que no presente se restringem à esfera do hipotético, uma vez que a sua potencial toxicidade para espécies aquáticas permanece por explorar. Esta preocupação ganha mais força se considerarmos que várias espécies aquáticas possuem recetores prostanóides filogeneticamente conservados.

Assim, surge a hipótese de toxicidade circular: o ser humano, quando num estado de stress oxidativo, vai excretar através da urina isoprostanos e estes, não sendo removidos ou degradados em estações de tratamento de águas residuais, irão entrar no ecossistema aquático e podem interagir com as múltiplas espécies possuidoras de recetores que os reconhecem. Entre os vários organismos aquáticos que são oportunos considerar neste contexto, destacam-se os invertebrados do Género *Daphnia*. Comummente conhecida por "pulga de água", trata-se de um grupo de crustáceos que troficamente atuam como consumidores primários, ocupando assim uma importante posição na cadeia trófica. Sob o ponto de vista toxicológico, são organismos resistentes à acção de químicos, mas simultaneamente sensíveis a pequenos incrementos nas concentrações dessas substâncias. Por estas razões, assumem elevada importância como bioindicadores da qualidade de água, sendo considerados por Instituições de referência (e.g.,

OCDE, ASTM) como organismos modelo no contexto da investigação em Ecotoxicologia. Adicionalmente, são fáceis de manter em laboratório, apresentam ciclos de reprodução curtos e as suas ninhadas são geneticamente iguais, isto é, os neonatos são clones do progenitor. Para além das evidentes vantagens, o ciclo de vida curto, permite avaliar efeitos de contaminantes ao longo de várias gerações, permitindo avaliar os efeitos em indivíduos que nunca foram expostos a um dado contaminante, mas que uma das suas gerações progenitoras esteve exposta.

Nesta tese, o principal objetivo passa por responder à pergunta "qual a ecotoxicidade associada aos efetores de stress oxidativo?". Para responder a esta pergunta, foram realizados testes de toxicidade aguda, crónica, multigeracional e transgeracional no organismo modelo *D. magna*. Os efetores de stress oxidativo testados foram o hipoclorito de sódio e os isoprostanos, tendo sido avaliados os seguintes parâmetros: sucesso reprodutivo nos vários testes, tempo para a primeira ninhada, imobilização dos neonatos, tamanho (comprimento) das dáfnias cronicamente expostas e mortalidade das dáfnias progenitoras ao longo de todos os testes.

Dos resultados obtidos foi possível concluir que a exposição a concentrações baixas de hipoclorito de sódio (0.045 e 0.09 $mg.L^{-1}$) afetou positivamente as dáfnias, uma vez que apresentaram maior número de neonatos assim como um maior tamanho, enquanto que a exposição a elevadas concentrações de hipoclorito de sódio (0.18, 0.36 e 0.72 $mg.L^{-1}$) afetou negativamente tanto o sucesso reprodutivo como o tamanho das dáfnias cronicamente expostas. Para além disso, foi possível concluir-se que o hipoclorito de sódio induz efeitos multigeracionais e/ou transgeracionais, nomeadamente ao nível do número de neonatos ao longo das várias gerações.

Relativamente aos isoprostanos, os efeitos ecotoxicológicos foram dependentes da concentração, com menor sucesso reprodutivo para a concentração mais elevada (1.6 $\mu g.L^{-1}$). No entanto, o sucesso reprodutivo para esta concentração é relativamente superior ao sucesso reprodutivo para a concentração superior de hipoclorito de sódio (0.72 $mg.L^{-1}$), quando normalizados para o respetivo controlo. Contrariamente ao hipoclorito de sódio, a exposição a isoprostanos não afetou o tamanho das dáfnias, sendo que independentemente da concentração, o seu comprimento foi sempre aproximadamente o mesmo. Por fim, foi possível concluir que os isoprostanos induziram efeitos multigeracionais, refletidos no número de neonatos obtidos.

De uma forma geral, foi possível demonstrar que marcadores de stress oxidativo, como os isoprostanos, podem também ser indutores de stress oxidativo em organismo aquáticos, permitindo desta forma validar a nossa hipótese inicial de toxicidade circular.

Abstract

In a state of redox homeostasis, an oxidative challenge initiates a stress response, executed through molecular redox switches, activating gene expression counteracting the challenge. Levels of oxidants and antioxidant species are balanced at a physiological level permitting redox signaling and redox regulation. However, certain stressors can elicit a response that unbalances this homeostatic state, inducing an imbalance between oxidants and antioxidants in favor of the oxidants, and leading to the prevalence of supraphysiological oxidative stress, disrupting redox signaling and/or damaging biomolecules, which may ultimately lead to the onset of disease. When the damaged biomolecules are lipids, the phenomenon is termed lipid peroxidation. The main target of lipid peroxidation are polyunsaturated fatty acids that, when oxidized, generate non-enzymatically biomolecules called isoprostanes. Thus, isoprostanes are considered final products of lipid peroxidation and, therefore, possible indicators of the existence of a state of oxidative stress associated with several pathologies.

Given that isoprostanes are excreted through urine, they can be detected in effluents from wastewater treatment plants and therefore be used as indicators in the assessment of the health status of a population. However, the fact that isoprostanes are detected in effluents can raise concern for the aquatic environment as their potential toxicity to aquatic species remains to be explored. Thus, we suggest the hypothesis that isoprostanes can initiate a phenomenon of circular toxicity: humans, when in a state of oxidative stress, will excrete isoprostanes through urine that is discharged in the sewage system and will reach WWTPs. Because most WWTPs lack the technology to completely remove or degrade these contaminants they will enter the aquatic ecosystem where they can interact with several species that have receptors that recognize them.

To test this circular toxicity hypothesis, the toxicity of effectors of oxidative stress (sodium hypochlorite and isoprostanes) was evaluated using standard acute and chronic toxicity tests performed using the model organism *Daphnia magna*. Furthermore, multigenerational and transgenerational tests were also performed to understand if these compounds induce effects in generations that aren't chronically exposed to them.

From the obtained results it is possible to conclude that when daphnids were chronically exposed, both to sodium hypochlorite and isoprostanes, reproductive success was dependent on the concentration. However, only sodium hypochlorite affected the size of daphnids, suggesting that the mechanism of action of these two compounds is different. In addition, it was also possible to conclude that both compounds are capable of inducing multigenerational and transgenerational effects on reproductive success, thus backing up our initial hypothesis of circular toxicity.

Keywords

Oxidative stress, Lipid peroxidation, Isoprostanes, *Daphnia magna*, Circular toxicity.

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AA – Arachidonic acid

AdA – Adrenic acid

COX – Cyclooxygenases

DHA – Docosahexaenoic acid

EPA – Eicosapentaenoic acid

IsoP – Isoprostane

LA – α-linolenic acid

OECD – Organisation for Economic Co-operation and Development

PG – Prostaglandin

PUFA – Polyunsaturated fatty acid

WBE – Wastewater-based epidemiology

WWTP – Wastewater treatment plants

Chapter I - Introduction

1.1. Oxidative stress

In homeostatic conditions, there is an equilibrium in the redox status. However, certain stressors can elicit a response that disrupts this homeostatic state, inducing an imbalance between oxidants and antioxidants, in favour of the oxidants. This leads to the prevalence of supraphysiological oxidative stress, disrupting redox signalling and/or damaging biomolecules, which may ultimately lead to the onset of disease (Pisoschi and Pop, 2015). In general terms, oxidative stress occurs when there is an imbalance in the regulation (control and signalling) of redox reactions in a biological system, with a higher prevalence of pro-oxidants than antioxidants (Lushchak, 2014; Pisoschi and Pop, 2015; Sies, 2015). The term pro-oxidant corresponds to any endobiotic or xenobiotic capable of inducing oxidative stress, either by generating free radicals or affecting the antioxidant response efficacy (Rahal et al., 2014). The oxidative damage that occurs is mainly attributed to free radicals, which may originate from several reactive species such as reactive oxygen species, and, to a lesser extent, reactive species of nitrogen, carbon, copper, halogens, iron and sulfur (Lushchak, 2014; Pisoschi and Pop, 2015). Free radicals are chemical species capable of existing independently that have an unpaired valence electron in their external orbit becoming highly unstable, and consequently very reactive, having short lifetimes (Lushchak, 2014; Pisoschi and Pop, 2015). Avoiding contact with free radicals is practically impossible, since besides being originated from the action of external agents, they are also generated endogenously by several metabolic processes, including cellular respiration, apoptosis and cellular differentiation. Among the exogenous stressors that can originate free radicals are ultraviolet radiation or compounds present, for example, in alcoholic beverages and tobacco smoke. Exposure to environmental contaminants as, for instance, pesticides also contributes to the free radicals loading (Pisoschi and Pop, 2015). As stated above, there are several types of free radicals (originated from different reactive species), and these include, among others, superoxide radical anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), nitric oxide ($\cdot NO$), alkoxyl radical ($RO\cdot$) and peroxyl radical ($ROO\cdot$). Furthermore, these free radicals can form or be produced with reactive species like hydrogen peroxide (H_2O_2), hydroxyl anion (OH^-), hypochlorous acid ($HClO$) and peroxynitrite ($ONOO^-$) (Genestra, 2007; Kalyanaraman, 2013; Pisoschi and Pop, 2015). The characteristics of these compounds, in the context of oxidative stress, as well as the reactions in which they're involved are described in Table 1.

Table 1 – Characteristics and reactions in which reactive species and free radicals are involved (information compiled from (Genestra, 2007; Kalyanaraman, 2013; Pisoschi and Pop, 2015)).

	Compound	Chemical formula	Characteristics	Reactions
Reactive species	Hydrogen peroxide	H_2O_2	Powerful liposoluble oxidant; Precursor and product of free radicals; Reacts with metal ions (Fenton reaction)	$\mathbf{H_2O_2} + e^- \rightarrow OH^- + HO\cdot$ $\cdot O_2^- + e^- + 2H^+ \rightarrow \mathbf{H_2O_2}$ $M^{n+} + \mathbf{H_2O_2} \rightarrow M^{(n+1)+} + HO\cdot + OH^-$
	Hydroxyl anion	OH^-	Product of Fenton reaction and H_2O_2 reduction	$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO\cdot + \mathbf{OH^-}$ $H_2O_2 + e^- \rightarrow \mathbf{OH^-} + HO\cdot$
	Hypochlorous acid	HClO	Induces oxidative chlorination on several biomolecules; High reactivity and liposolubility; Formed through the action of myeloperoxidase	$H_2O_2 + Cl^- \rightarrow \mathbf{HClO} + OH^-$
	Peroxynitrite	$ONOO^-$	Strong oxidant and nitrating agent; Liposoluble and capable of damaging several biomolecules	$\cdot O_2^- + \cdot NO \rightarrow \mathbf{ONOO^-}$ $\mathbf{ONOO^-} + H^+ \rightarrow HO\cdot + \cdot NO_2$
Free radicals	Hydroxyl radical	·OH	Very short *in vivo* half-life, highly damaging and reactive towards most biomolecules; Formed from Fenton reaction, H_2O_2 reduction and $ONOO^-$ decomposition	$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + \mathbf{HO}\cdot + OH^-$ $H_2O_2 + e^- \rightarrow OH^- + \mathbf{HO}\cdot$ $ONOO^- + H^+ \rightarrow \mathbf{HO}\cdot + \cdot NO_2$
	Superoxide radical anion	$\cdot O_2^-$	Less reactive when compared to ·OH; Involved in the production of reactive species	$O_2 + e^- \rightarrow \cdot \mathbf{O_2}^-$ $\cdot \mathbf{O_2}^- + e^- + 2H^+ \rightarrow H_2O_2$ $\cdot \mathbf{O_2}^- + \cdot NO \rightarrow ONOO^-$
	Nitric oxide	·NO	Can be synthesized from L-arginine, oxygen and NADPH; Released by phagocytes	$\cdot O_2^- + \cdot \mathbf{NO} \rightarrow ONOO^-$
	Alkoxyl radical	RO·	Organic radicals that, when in the form of lipids, take part in lipid peroxidation	With oxygen, produced either by radical addition to the double bond or by hydrogen removal
	Peroxyl radical	ROO·		

From all of the free radicals presented, $\cdot O_2^-$ and ·OH arguably deserve more attention due to the fact that their generation occurs mainly during aerobic respiration, i.e., they are formed when molecular oxygen is reduced in ubiquitous aerobic processes. Normally, the reduction of oxygen to water occurs in a direct way that leads to the production of chemical energy in the form of ATP, without the production of free radicals. However, if oxygen is reduced one electron at a time, free radicals are formed as intermediate products and only after four reductions water is generated (Kalyanaraman, 2013; Lushchak, 2014). The possible reductions of oxygen are shown in Figure 1.

$$a) \quad O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$$

$$b) \quad O_2 \xrightarrow[e^-]{} \cdot O_2^- \xrightarrow[e^-]{2H^+} H_2O_2 \xrightarrow[e^-]{} HO \cdot + OH^-$$

$$b1) \quad HO \cdot + e^- + H^+ \longrightarrow H_2O$$

$$b2) \quad OH^- + H^+ \longrightarrow H_2O$$

Figure 1: Reduction of molecular oxygen. Regardless of the type of reduction, the balance of reagents and products are the same. a) Oxygen is directly reduced to water; b) Oxygen is reduced step-by-step, in which intermediates (free radicals) are produced; b1) and b2) Last reduction step where ·OH and OH- originate H_2O (Kalyanaraman, 2013; Lushchak, 2014).

Ultimately, some of the free radicals mentioned above can be the factor that triggers certain changes at cellular level, which in the long run may be responsible for the pathogenesis of several diseases like cardiovascular, neurodegenerative, endocrine, inflammatory, kidney and oncological diseases (Pisoschi and Pop, 2015). However, it is important to stress that free radicals aren't exclusively negative agents. Although their effects are deleterious to several biomolecules and, as such can have a negative influence in the aging of cells (eventually leading to their improper functioning), when present at low concentrations, they are used as signalling molecules in several important biologic processes like apoptosis, gene expression and regulation of cell proliferation and therefore are beneficial (Lushchak, 2014; Pisoschi and Pop, 2015).

The counter part of free radicals are antioxidants, compounds capable of preventing or reducing oxidative damage, and therefore, their purpose being to maintain redox homeostasis (Kalyanaraman, 2013; Pisoschi and Pop, 2015). Antioxidants can be classified in several ways and, in Table 2, two types of classification are considered: enzymatic/non-enzymatic and endogenous/exogenous antioxidants.

Table 2 – Classification and characteristics of several antioxidants (information compiled from (Carocho and Ferreira, 2013; Pisoschi and Pop, 2015)).

Classification		Antioxidant	Function/ Characteristics
Enzymatic	Endogenous	Catalase	Reduces H_2O_2 to water and molecular oxygen
		Glutathione peroxidase	Reduces H_2O_2 and organic hydroperoxides to water or its respective alcohol. The reduction of peroxides forms selenoles
		Superoxide dismutase	Converts $\cdot O_2^-$ to H_2O_2
		Glutathione reductase	When oxidized reduces glutathione, making it newly available to reduce free radicals
		Glucose-6-phosphate dehydrogenase	Regenerates NADPH which, subsequently, is used to reduce glutathione reductase
Non-enzymatic	Endogenous	Coenzyme Q10	Cofactor that intervenes in cellular respiration by carrying electrons. Prevents lipid ROO· and has the ability to regenerate vitamin E
		Glutathione	Organosulfur compound that donates hydrogen or electrons (when its thiol groups are oxidized to disulphide bonds). It can also regenerate vitamin C
		Uric acid	Important nitrogen non-protein scavenger of ·OH and peroxyl radicals and singlet oxygen
		Vitamin A - retinol	Origins from ß-carotene and avoids ROO· induction of lipid peroxidation
	Exogenous	Vitamin C - ascorbate	Scavenger of H_2O_2, $\cdot O_2^-$, ·OH, singlet oxygen and nitrogen oxide. Presents synergistic effect with vitamin E in lipid peroxidation
		Vitamin E - tocopherols	Capable of breaking the chain oxidations in lipid peroxidation
		Vitamin K	Protective character due to the presence of 1,4-naphthoquinone in its structure
		Metallothioneins	Thiol group in their cysteine residues can bind to ionic metals and cysteine residues, by themselves, can capture $\cdot O_2^-$ and ·OH
		Selenium	Doesn't have direct contact with free radicals but is an important part of glutathione peroxidase and metalloenzymes
		Zinc	Acts indirectly in the prevention of free radicals generation by being a part of superoxide dismutase and inducing production of metallothioneins
		Flavonoids	Large group of compounds that have in common a diphenylpropane skeleton. Phenolic hydroxyl groups attached to this skeleton give these compounds the ability as reducing agents, hydrogen donators and $\cdot O_2^-$ scavengers
		Phenolic acids	Consist of hydroxycinnamic and hydroxybenzoic acids which can act as chelators and scavengers of ·OH, ROO·, $\cdot O_2^-$ and $ONOO^-$
		Carotenoids	Group of pigments that are able to quench singlet oxygen and destroy ROO·

Enzymatic antioxidants, all of them endogenous antioxidants, can be further subdivided into primary and secondary enzymatic defences. The primary defences are composed of catalase, glutathione peroxidase and superoxide dismutase whose general purpose is to prevent both the formation of free radicals and to neutralize them. The secondary enzymatic defences, in which glutathione reductase and glucose-6-phosphate dehydrogenase are included, have as main goal the support of other endogenous antioxidants. Non-enzymatic endogenous antioxidants include coenzyme Q10, glutathione, uric acid and vitamin A and their role is basically to scavenge for

free radicals (Carocho and Ferreira, 2013). However, in some situations, the endogenous antioxidant's response is not sufficient to counterbalance the effects of oxidative stress, and in such cases the redox homeostasis is disrupted.

Non-enzymatic antioxidants (practically all of them exogenous), are particularly important when the increase in oxidative stress is a consequence, for example, of exposure to exogenous free radicals or of aging. Comprising several compounds from different classes, they work as an additional source that is able to re-establish the adequate levels of antioxidants in a situation of oxidative stress and are preferentially used over endogenous antioxidants (Carocho and Ferreira, 2013; Pisoschi and Pop, 2015). In this way, the action of endogenous antioxidants is complemented with exogenous antioxidants in the quest to maintain their levels balanced relatively to pro-oxidants, thus avoiding the occurrence of oxidative stress. However, and not ignoring the important role that all antioxidants play, it is pertinent to question if they are always beneficial. In fact, there are situations where antioxidants can assume the role of a pro-oxidant. As examples, we can refer (1) vitamin C that, when combined with metal ions, reduces Fe^{3+} or Cu^{3+} to Fe^{2+} or Cu^{2+} and can reduce H_2O_2 to ·OH; (2) the lack of regeneration of vitamin E when reacting with free radicals under insufficient levels of ascorbic acid; and lastly, (3) the generation of ·OH by carotenoids undergoing autoxidation in the presence of high concentrations of ROS (Carocho and Ferreira, 2013). Additionally, we can also hypothesize if it is possible that antioxidants, like pro-oxidants, can deregulate redox homeostasis and confer a state of imbalance between these but favourable to antioxidants? This possible deregulation by antioxidants is mainly associated with exogenous antioxidants from food sources and is thoroughly discussed by Carocho and Ferreira (2013).

Controversies associated with the role of pro-oxidants and antioxidants include, among others: (i) heterogeneity in the results obtained for the same antioxidant, in the sense that different outcomes were obtained from different studies for the same antioxidant and thus they can be considered as "double-edged swords" (see Bouayed and Bohn (2010) for a complete list of references); (ii) the effect of pro-oxidants is not only necessary for several important biological processes, it is also suggested that, when capable of creating a slight state of oxidative stress, this leads to increased levels of antioxidants, creating a situation of cytoprotection in the body. For all this, and in the same way as pro-oxidants, we can rationalize that the contribution of antioxidants is important and extremely necessary. Yet again, it is essential that they remain within the desired levels in order not to compromise their respective delicate dynamic balance (Carocho and Ferreira, 2013). Therefore, in order to achieve the desired state of homeostasis, there must be a dynamic equilibrium state between pro-oxidants and antioxidants. For example, in a case of basal oxidative stress, is hard to say for sure if it's part of the dynamic equilibrium or if it could be the beginning of a potential situation of oxidative stress. Overall, it is difficult to define beyond doubt when the dynamic equilibrium no longer exists and gives rise to a state of oxidative stress (Lushchak, 2014).

1.2. Lipid peroxidation – isoprostanes synthesis and classification

The interaction of the free radicals with biomolecules like DNA, proteins and lipids can lead to their mutation, oxidation and peroxidation, respectively, with these changes being involved in the pathogenesis of several diseases (Milne et al., 2011). Lipid peroxidation consists in the interaction of free radicals with lipids which, upon oxidation, will consequently propagate a series of lipidic oxidations until this chain reaction is interrupted. Therefore, it translates as a consequence of oxidative stress and can thus be used as an indicator of its occurrence (Ayala et al., 2014; Kalyanaraman, 2013; Milne et al., 2011). This process occurs in three phases, namely initiation, propagation and termination as depicted in Figure 2.

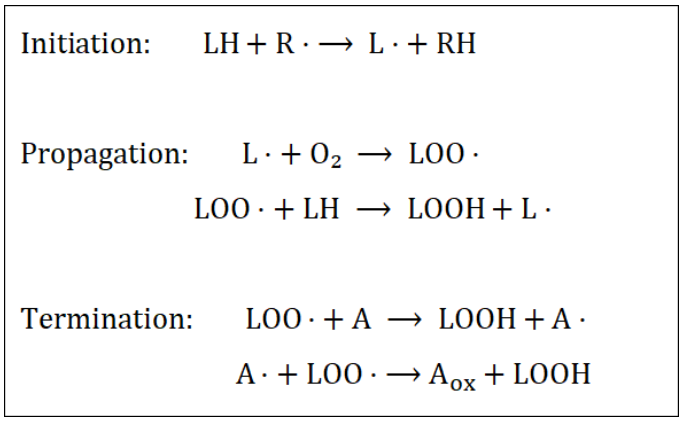

Figure 2: Steps of lipid peroxidation. **LH**: lipid; **R·**: free radical; **L·**: lipid radical (oxidized); **RH**: reduced radical; **LOO·**: lipid peroxyl radical; **LOOH**: lipid hydroperoxide; **A**: antioxidant; **A·**: antioxidant radical; **A_{ox}**: oxidized antioxidant (stable). Adapted from Kalyanaraman (2013).

The first step of lipid peroxidation (initiation) occurs when free radicals oxidize lipids, causing them to have a carbon-centered radical. However, this lipid radical suffers a molecular rearrangement in order to become more stable, yielding a conjugated diene. In the propagation phase, the lipid radical will rapidly react with oxygen, giving rise to a lipid peroxyl radical. It is due to this newly formed compound that the whole process of lipid peroxidation will assume its character of a continuous chain of reactions: the lipid peroxyl radical will remove a hydrogen from another lipid, oxidizing it, which generates, respectively, a lipid hydroperoxide and a new lipid radical. This series of oxidations will continue until this propagation phase is interrupted. The interruption of this phase only occurs by the action of antioxidants. In the termination phase, antioxidants donate a hydrogen to the lipid peroxyl radical, and the "antioxidant radical" generated will then react with another lipid peroxyl radical, giving rise to non-radical products (oxidized antioxidant and lipid hydroperoxide) and thus ending the lipids' peroxidation (Ayala et al., 2014; Kalyanaraman, 2013). This type of response towards lipid peroxidation can be regarded as a response of the cells in order to promote their survival when facing low levels of lipid peroxidation at subtoxic conditions. However, if the lipid peroxidation is overwhelming,

i.e. if it reaches toxic conditions, cells will not try to solve this situation and apoptosis will be induced (Ayala et al., 2014).

The main targets of lipid peroxidation are polyunsaturated fatty acids (PUFAs). This happens because PUFAs have bis-allylic methylene groups (-CH_2- that are flanked by two double bounds) in their structure. Consequently, PUFAs will have higher reactivities, under physiological conditions, than saturated and mono-unsaturated fatty acids. Therefore, PUFAs like arachidonic acid (AA), adrenic acid (AdA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and α-linolenic acid (LA) will be targeted by free radicals in a cascade mechanism that culminates in cellular membrane damage and changes in membrane characteristics (Jahn et al., 2008; Milne et al., 2011). The differences between the above mentioned PUFAs lie on the number of bis-allylic methylene groups each one possesses: LA has two, AA and AdA have three, EPA has four and DHA has five. These characteristics will affect the easiness of PUFAs oxidation in a directly proportional way, and so, the oxidation susceptibility occurs as follows: LA < AA ≈ AdA < EPA < DHA (Milne et al., 2011; Niki, 2014). Figure 3 illustrates the mentioned PUFAs as well as the location of their respective bis-allylic methylene groups.

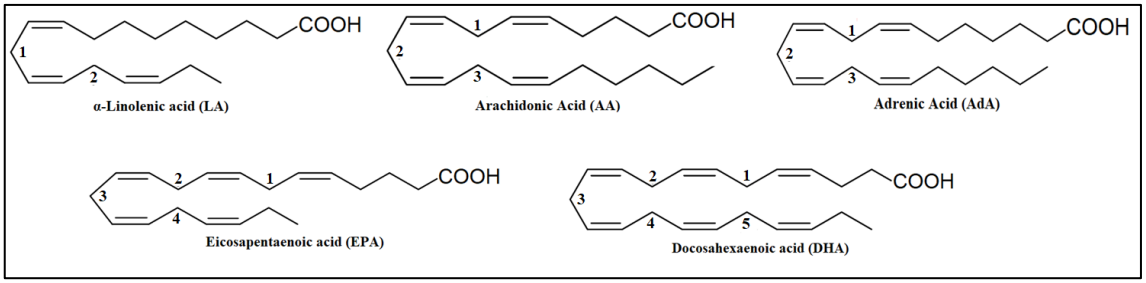

Figure 3: PUFAs. Adapted from Galano et al. (2017).

These PUFAs are involved as substrates in various enzymatic and non-enzymatic reactions that give rise to a great diversity of important biologically active molecules that participate in signalling, mediation and act as secondary metabolites (Jahn et al., 2008). When subjected to lipid peroxidation, i.e. oxidized, they generate a class of prostaglandin-like compounds that are produced non-enzymatically known as isoprostanes (IsoPs) (Milne et al., 2015). Although similar, since both prostaglandins (PGs) and IsoPs are cyclic compounds derived from PUFAs, there are two distinctive features between these biomolecules (Jahn et al., 2008). The first one is associated with their synthesis. PGs production is associated to tissue inflammation and are synthesized when AA is released from phospholipids (by the action of phospholipase A_2) and afterwards metabolized by cyclooxygenases (COX) (Ricciotti and FitzGerald, 2011; Yen et al., 2015). IsoPs result of peroxidation induced by free radicals in PUFAs (lipid peroxidation) (Milne et al., 2011). Second, PGs and IsoPs differ structurally in their conformation, i.e., in their stoichiometry: whereas the two PG side chains have a *trans* conformation relative to the cyclopentane ring (thermodynamically favourable), the two side chains of IsoPs have a *cis* conformation (kinetically favourable) (Daughton, 2012). The nomenclature of IsoPs, similiary

to PGs, is determined by the functional groups and their respective arrangement in the cyclopentane ring (Milne et al., 2011) and, in this document, the nomenclature system used is the one proposed by Taber and co-workers (Taber et al., 1997). The example in Figure 4 illustrates the described differences regarding their synthesis and stoichiometry, as well as an example of how one obtains the respective correct name using this nomenclature system.

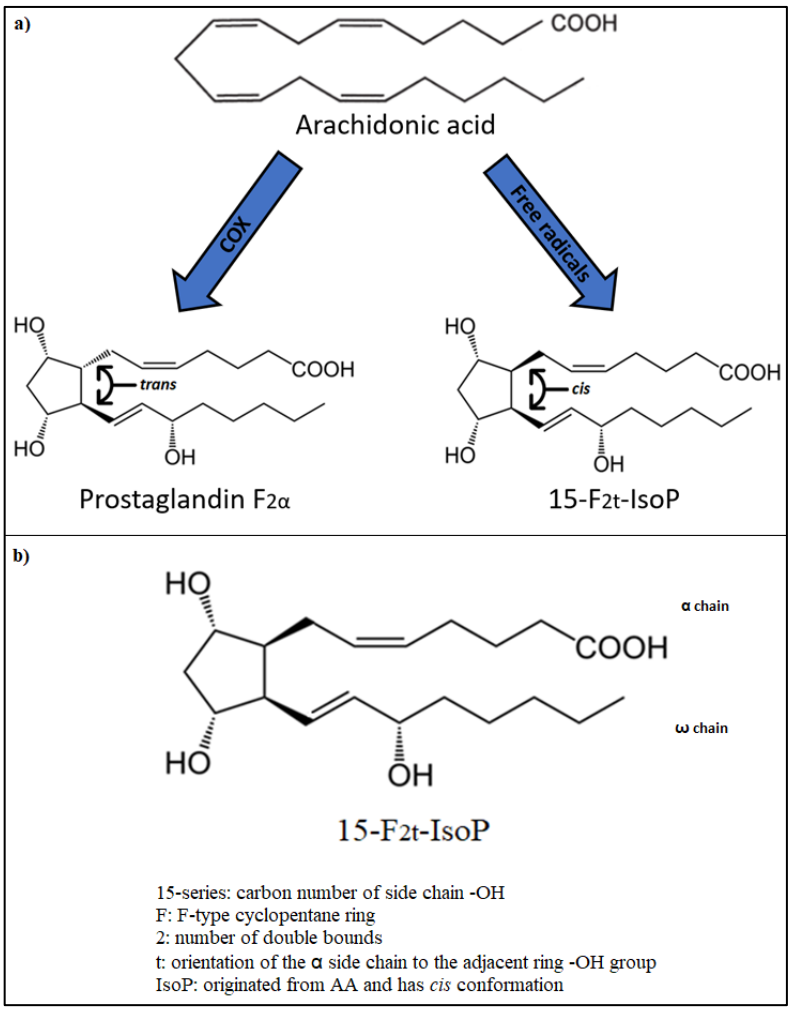

Figure 4: a) Difference between PGs ($F_{2\alpha}$-PG) and IsoPs (15-F_{2t}-IsoP). **b)** Nomenclature example of 15-F_{2t}-IsoP according to Taber and co-workers system (Jahn et al., 2008; Taber et al., 1997).

Naturally, AA isn't the only PUFA that generates an IsoP when oxidized. Taking into account the PUFAs previously discussed and using as an example IsoPs that all have a F-type cyclopentane ring, the following different IsoPs are obtained: F_2-IsoPs are the oxidation product of AA, F_2-dihomo-IsoPs generate from AdA, F_3-IsoPs result from EPA, F_4-neuroprostanes are obtained from DHA and phytoprostanes are the product of LA (Jahn et al., 2008; Milne et al., 2011; Niki, 2014; Yen et al., 2015). From the previous example, the most studied and discussed IsoPs are probably the ones generated from AA, being F_2-, D_2- and E_2-IsoPs the main classes to be considered (Jahn et al., 2008; Milne et al., 2011). Of these three isomers, F_2-IsoPs are the most stable when compared to D_2- and E_2-IsoPs because these two are dehydrated to J_2- and

A_2-IsoPs, respectively (Milne et al., 2011). Again, just like PGs, F_2-IsoPs also have several different isomers (up to 64 are possible), depending on where the oxidation of AA occurs (Gaw and Glover, 2016; Yen et al., 2015). This number of isomers is possible because, when AA is subject to lipid peroxidation by free radicals, it produces four regioisomers and each of these will, in turn, give rise to eight additional isomers together with their respective diastereomers (Daughton, 2012). The four regioisomers have a classification to discriminate them from each other, taking into account the carbon where the hydroxyl group is and starting counting from the carbon of the carboxyl group. By doing this, hydroxyl groups can be bound to different carbons which occupy positions 5, 8, 12 or 15, and, therefore, F_2-IsoPs will be present in the form of 5-F_2-IsoPs, 8-F_2-IsoPs, 12-F_2-IsoPs and 15-F_2-IsoPs, respectively. It is important to note that the regioisomers of the 5- and 15-series are formed at significantly higher amounts than the regioisomers of the 8- and 12-series because the latter are still susceptible to oxidation, yielding a type of compounds called dioxolane-IsoPs. Thus, since regioisomers of series 5- and 15-series are not oxidized, they correspond to the final products of the overall oxidation reaction (Milne et al., 2011). Figure 5 illustrates some IsoPs examples that PUFAs can origin, as well the contextualization of the IsoPs that origin particulary from AA. With all this said about IsoPs, one can think that they are undoubtedly only generated by lipid peroxidation. However, this traditional idea that IsoPs generation is enzyme independent isn't entirely true (Milne et al., 2011). Recently, some authors have been questioning the established paradigm that IsoPs formation is independent of enzymatic action. van't Erve et al. (2016) added important evidence that IsoPs are not solely products of oxidation induced by free radicals (independent of enzymes) but can also be obtained through enzymatic oxidation via COX, just like PGs. In practical terms, these authors highlighted that, in order to better characterize the real contribution of oxidative stress, one should examine the ratio of a specific IsoP to its respective PG generated by COX: the enzymatic pathway is responsible for a higher proportion of the latter over the former, whereas the proportion is similar when oxidative stress is the mechanism underlying the formation of these compounds (van't Erve et al., 2016).

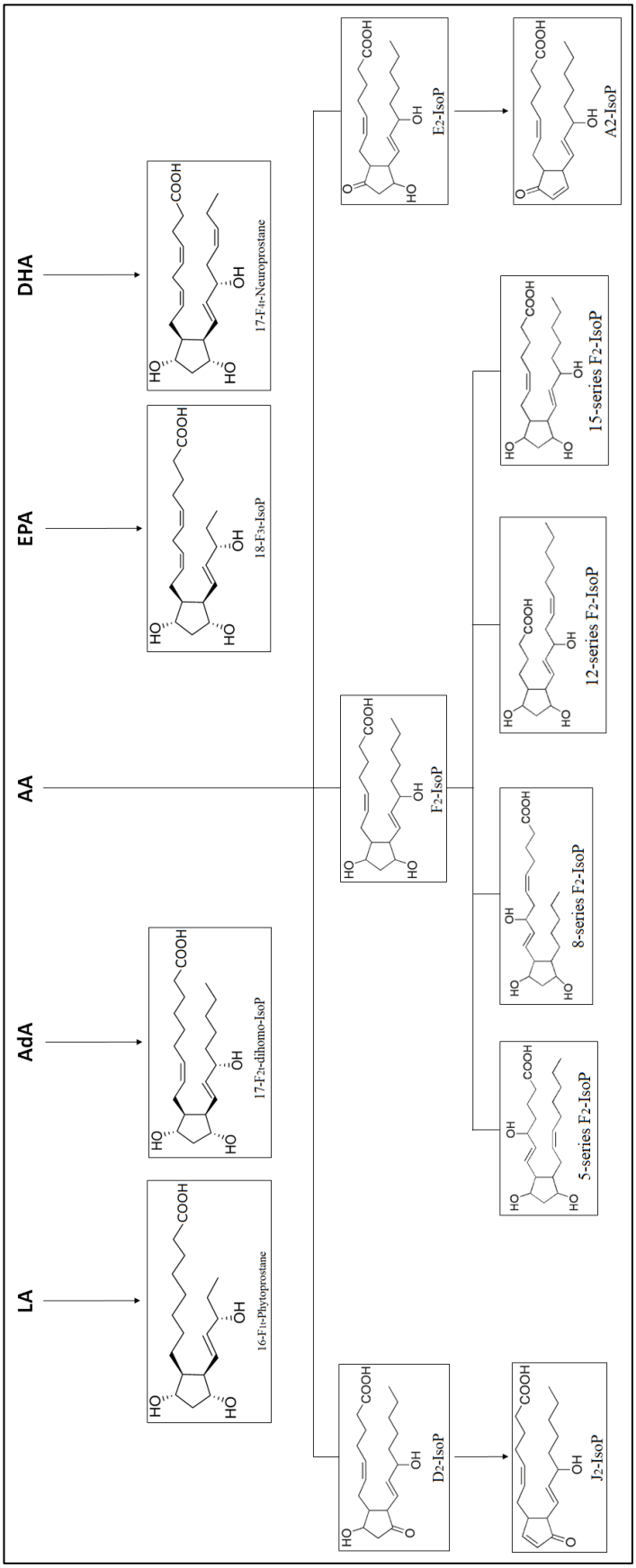

Figure 5: Contextualization of F-type IsoPs from different PUFAs and IsoPs generated from AA. Images adapted from (Galano et al., 2017; Milne et al., 2011).

1.2.1. Isoprostanes as markers of oxidative stress

The production of IsoPs, and their respective cleavage by phospholipases, occurs in the membrane lipids that constitute all tissues of living organisms (Gaw and Glover, 2016). Generally, 15-F_2-IsoPs (also known as 8-iso-$PGF_{2\alpha}$) are the most abundant F_2-IsoPs. They can be detected in plasma (free IsoPs) and urine (free and glucuronide-conjugated IsoPs) and thus they can be used as clinical biomarkers of oxidative stress (Gaw and Glover, 2016; Milne et al., 2015). Morrow et al. (1990) established for the first time the normal levels of F_2-IsoPs both for plasma (0.005-0.04 ng/mL) and in urine (0.5-4 ng/mg creatinine). Later, Liu et al. (1999) reported that the normal levels were 0.035 ± 0.006 ng/mL for human plasma and 1.6 ± 0.6 ng/mg creatinine for urine. More recently, van 't Erve et al. (2017), reported urinary levels of 1.2 ± 0.6 ng/mL (or 1.3 ± 0.8 ng/mg creatinine) in control individuals and plasma levels of 0.0451 ± 0.0184 ng/mL. Because urinary levels of free 15-F_2-IsoPs are much higher than those in plasma, urine is currently the preferred matrix to quantify F_2-IsoPs, with the additional advantage of being non-invasive, which facilitates its collection. In addition to plasma and urine, other matrices such as amniotic fluid, central nervous system samples (brain tissue, cerebrospinal fluid, anterior cingulate cortex, occipital cortex), atherosclerotic plaques, carotid plaques, cord serum, exhaled breath condensate, proximal gastric mucosa, saliva, serum and sputum, may be used, as comprehensibly described by Galano et al. (2017) and illustrated in Figure 6; however, their use is still limited.

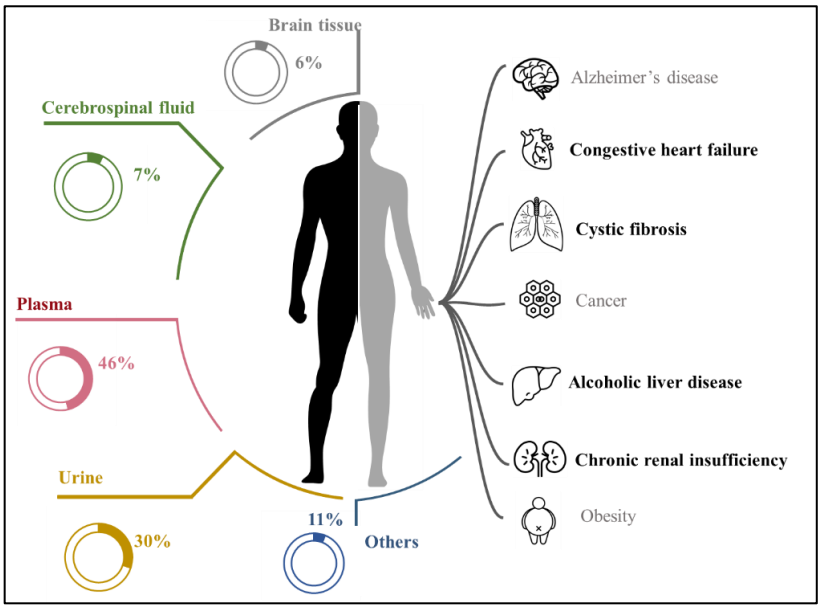

Figure 6: Diagram illustrating the matrices used to monitor the levels of IsoPs and the diseases for which IsoPs were traditionally considered good biomarkers (depicted in grey) and the diseases for which the new scientific evidences suggests stronger associations with IsoPs levels (depicted in black, bold letters). "Others" matrices refer to amniotic fluid, atherosclerotic plaques, carotid plaques, cord serum, exhaled breath condensate, anterior cingulate cortex, occipital cortex, proximal gastric mucosa, saliva, serum and sputum. Data extracted from Galano et al. (2017), Milne (2017) and van 't Erve et al. (2017).

The use of F_2-IsoPs as biomarkers for several diseases was recently reviewed by Galano et al. (2017). On this basis, levels of all F_2-IsoPs, 15-F_2-IsoPs and its metabolites, and even 5-F_2-IsoPs, have been quantified in individuals with different diseases, aiming to associate the levels of IsoPs with pathologies known to be associated with lipid peroxidation and, thus, with oxidative stress. Overall, there are some pathologies for which the use of IsoPs as biomarkers is more consensual than others. From the data compiled by Galano et al. (2017), we can infer that IsoPs are considered good biomarkers for: liver diseases (alcoholic liver disease, autoimmune hepatitis, biliary cirrhosis and non-alcoholic fat liver disease), stroke, asthma, thalassemia, dengue fever, sepsis (related to renal, hepatic and coagulation failure), periodontal disease, oral lichen planus, critically ill patients, community acquired pneumonia, paediatric sepsis, *Helicobacter pylori* infection, obstructive sleep apnoea, asymptomatic/symptomatic atherosclerosis and coronary/peripheral artery disease. For all of these diseases, F_2-IsoPs were higher in patients than in controls and therefore indicated a disease associated with oxidative stress (Galano et al., 2017). However, the seemingly direct relation between the diseases listed above and the presence of higher levels of IsoPs should be taken with a grain of salt: almost all of the above pathologies were addressed in one single study and different approaches (different matrices and/or different quantification methods) can contradict the first results obtained.

Neurological diseases are the ones less consensual, as the levels of F_2-IsoPs between patients and controls don't follow any tendency. For Alzheimer's and Parkinson's disease, both with a relatively high number of studies addressing them, different studies presented both higher and equal levels of F_2-IsoPs when patients are compared to controls. The same happened with schizophrenia, mild cognitive impairment, and traumatic brain injury. The pathologies that had a concordant increase of F_2-IsoPs in more than one study were epilepsy and autism (children). Regarding single study investigations, Huntington's disease, multiple system atrophy, dementia and Down syndrome register the same values of F_2-IsoPs, while brain injury (after aneurysmal subarachnoid hemorrhage), reversible cerebral vasoconstriction syndrome, intermittent explosive disorder, hypoxic-ischemic encephalopathy and neuroborreliosis were associated with an increase of F_2-IsoPs (Galano et al., 2017).

Regarding cardiovascular diseases, hypertension was the most studied, and discordant results of both higher and equal levels of F_2-IsoPs in relation to controls were reported. The remaining cardiovascular diseases (all one-case studies) of mild-moderate hypertension and heart failure didn't showed changes in its F_2-IsoPs levels. The cancer studies were limited, and thus it is difficult to ascertain if F_2-IsoPs levels are an indicator of this type of pathology. Nevertheless, from the four one-case studies analysed, prostate cancer and esophageal adenocarcinoma presented higher levels of F_2-IsoPs while breast cancer and uterine leiomyomas (fibroids) patients didn't have any change on their F_2-IsoPs levels. Diseases associated with diabetes have also been targeted, with type 2 diabetes mellitus (also one of the most addressed pathologies) registering higher, equal and even lower levels of F_2-IsoPs and metabolic syndrome presented both higher and equal levels of F_2-IsoPs. In this class, only type 1 diabetes mellitus presented

concordance between studies, in which F_2-IsoPs levels remained the same among patient and control groups. In renal dysfunction diseases, chronic kidney disease was the most addressed pathology and the only which didn't reunited consensus between studies, having both higher and equal levels of F_2-IsoPs. The remaining diseases and one-case studies were nephrotic syndrome and acute renal graft dysfunction, which had, respectively, same and higher levels of F_2-IsoPs. Finally, other diseases as preeclampsia, influenza A and rheumatoid arthritis also didn't reunite consensus about the change in F_2-IsoPs levels, with the last one even registering a decrease in patient levels (in plasma, free IsoPs decreased while esterified IsoPs increased) (Galano et al., 2017). Interestingly, it seems that the more a pathology is studied, the less consensus seems to exist, having different types of levels of F_2-IsoPs associated to it, as it is the case with Alzheimer's, Parkinson's and type 2 diabetes mellitus. Whatever may be the source of this variability, it is undeniable that the type of sample used, as well as the respective quantification method, will surely affect the outcome levels of F_2-IsoPs.

Traditionally, higher IsoPs levels were associated, for example, with neurodegenerative diseases, cancer and obesity as depicted in Figure 6. However, upon the paradigm change proposed by van't Erve et al. (2016) (see section 2) new evidences arose and the list of diseases associated with higher IsoPs levels was revised and updated (van 't Erve et al., 2017). These authors performed a meta-analysis in order to understand the role of oxidative stress markers (free and total 8-iso-$PGF_{2\alpha}$) across human disease and compiled the available data for free 8-iso-$PGF_{2\alpha}$ and total 8-iso-$PGF_{2\alpha}$. The difference between these is that total 8-iso-$PGF_{2\alpha}$ includes free 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ that is esterified to LDL phospholipids. Overall, the authors consider that the total 8-iso-$PGF_{2\alpha}$ is a better indicator of oxidative stress because, if only free 8-iso-$PGF_{2\alpha}$ is assessed, it can include the enzymatically generated 8-iso-$PGF_{2\alpha}$, and, therefore, compromise the correct interpretation of 8-iso-$PGF_{2\alpha}$ as a biomarker of oxidative stress. Thus, pathologies that have total 8-iso-$PGF_{2\alpha}$ levels higher than the free 8-iso-$PGF_{2\alpha}$ levels will, most likely, be generated non-enzymatically, as a consequence of oxidative stress. However, the number of publications with total 8-iso-$PGF_{2\alpha}$ levels (n=33) is much lower than the number of publications reporting free 8-iso-$PGF_{2\alpha}$ (n=209) (van 't Erve et al., 2017), most probably due to the difficulties associated with the quantification of total 8-iso-$PGF_{2\alpha}$ levels. Based on the results obtained from the meta-analysis, van't Erve and colleagues, established a new and more robust ranking of human pathologies in which lipid peroxidation has higher interference. Diseases of the respiratory tract such as cystic fibrosis, pulmonary arterial hypertension and urogenital diseases such as chronic renal insufficiency were recognized as those for which the extension of oxidative damage is higher. On the other hand, Alzheimer's disease, obesity and cancer, that were previously associated with significant oxidative stress, presented lower association with increased levels of 8-iso-$PGF_{2\alpha}$ (Milne, 2017).

1.3. Sodium hypochlorite as effector of oxidative stress

Sodium hypochlorite (NaClO) is an environmental contaminant that occurs in the aquatic environment mainly as a consequence of its widespread use as a disinfectant for drinking and recreational waters (Nimkerdphol and Nakagawa, 2008). It is also largely employed in hospital wastewaters due to it's effective biocidal properties. The versatile employment of NaClO in several contexts is due to the the fact that it easily reacts with water, originating hypochlorous acid (HClO – reactive species: see section 1.1, Table 1), which in turn will dissociates in aqueous medium, resulting in hypochlorite anion (ClO^-) (Emmanuel et al., 2004). The toxicity character will therefore be atributed to HClO, ClO^- and also chlorine.

Because of this, several aquatic organisms can and have been used to assess the acute toxicity of these chemical species. Some examples of acute toxicity assesment include the use of aquatic organisms like *Pimephales promelas* (Curtis and Ward, 1981), *Asellus intermedius, Daphnia magna, Dugesia tigrina, Gammarus fasciatus, Helisoma trivolvis, Lumbriculus variegatus* and *Pimephales promelas* (Ewell et al., 1986), *Daphnia similis*, *Oryzias latipes* and *Oreochromis mossambicus* (Chen et al., 2001) and *Vibrio fischeri, Daphnia magna, and Pseudokirchneriella subcapitata* (Pignata et al., 2012) for NaClO and *Ceriodaphnia dubia and Penaeus plebejus* (Manning et al., 1996), *Daphnia magna, Daphnia pulex, Nitocra spinipes, Oncorhynchus mykiss, Salvelinus fontinalis* and *Lepomis cyanellus* (Emmanuel et al., 2004) and *Ceriodaphnia silvestrii and Daphnia similis, Chironomus xanthus and Danio rerio* (da Costa et al., 2014) for chlorine. However, prior to all these, Brungs (1973) had already compiled several studies with diverse acute and chronic toxicity endpoints for chlorine with aquatic organisms such as *Salvelinus fontinalis, Salmo trutta, Oncorhynchus mykiss, Perca flavescens, Micropterus salmoides, Micropterus dolomieu, Catostomus commersonii, Sander vitreus, Ameiurus melas, Pimephales promelas, Notemigonus crysoleucas* and *Daphnia magna*. This serves to show that NaClO and its associated chemical species toxicity has always been a preocupation for aquatic ecossystems and their species.

Recently, acute toxicity tests have tried to include the use of some of the organisms previosly listed but with different acute toxicy endpoints for NaClO. Some examples include the use of resting eggs sensitivity of *Daphnia mendotae* (Raikow et al., 2007), the swimming behaviour of *Danio rerio* as a tool to be implemented in biomonitorization systems for acute toxicity bioassays (Nimkerdphol and Nakagawa, 2008) and the use of *Daphnia magna* to evaluate teratogenic effects (Ton et al., 2012). Although NaClO acute toxicity is well described for several freshwater organisms, its chronic effects are not well explored and more studies should be performed since NaClO is present in aquatic ecossystems and, thus, it is important to understand how its chronic exposure affects aquatic species, as well as how they cope with it.

1.4. *Daphnia* as model organisms

In almost all aquatic environments, except habitats with extreme conditions, small planktonic crustaceans of the order Cladocera and the genus *Daphnia* can be found (Ebert, 2005; Smirnov, 2017). These organisms are primary consumers, occupying an important position in the complex intertwined trophic interactions of aquatic ecosystems and thus are important bioindicators of water quality (Hasenbein et al., 2017; Smirnov, 2017). *Daphnia* are commonly known by "water flea" because their swimming behaviour is similar to a little hop, thanks to the beating of its antennae (Bownik, 2017; Ebert, 2005). The diet of a *Daphnia* is normally composed of phytoplankton, with green algae being their preferred food source, but their feeding behaviour isn't that discriminatory since they can consume particles in a range size of 1 µm – 50 µm (Ebert, 2005; Hasenbein et al., 2017). The life cycle of *Daphnia* (Figure 7) includes both asexual (cyclic parthenogenesis) and sexual reproduction.

In the particular case of cyclic parthenogenesis with the proper and favourable conditions of food, water, temperature, pH and photoperiod, an adult female will produce a clutch of variable number every time the female sheds its carapace (every 3 days). These parthenogenetic eggs will contain embryos which will be genetically identical to their progenitor. The neonates released, also all female, will be ready to produce their own clutches around 7-10 days after being born and thus the cycle repeats itself (Ebert, 2005). It is mainly due to some of these characteristics that daphnids are interesting model organisms for research purposes: they're relatively easy to maintain as a culture in a laboratory due to their small but visible size, they are transparent, which enable to visualize their internal organs, they have a short reproductive cycles and life span and, finally, their broods is genetically identical i.e. neonates are clones of their progenitor (Hasenbein et al., 2017). Because daphnids are, at the same time, sensitive and resilient, their use in ecotoxicological studies is well established and their acute and chronic toxicity evaluation is recommended by several regulatory agencies, including the Organisation for Economic Co-operation and Development (OECD). Standardized tests for both acute (OECD/OCDE, 2004) and chronic (OECD/OCDE, 2012) are required for regulatory compliance tests (e.g. effluents and leachates) and for the introduction of several substances into the market (e.g. under REACH regulation) (ECHA, 2017).

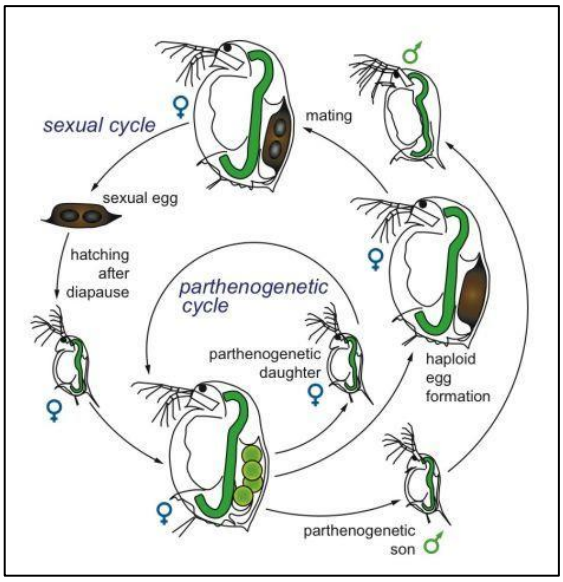

Figure 7: *Daphnia* life cycle. Adapted from Ebert (2005).

Besides the advantages referred above, due to their short life span, daphnids can also be used to evaluate long term, inter and transgenerational effects of environmental stressors. Recent evidence suggests that the negative effects of several contaminants (particularly endocrine disrupting chemicals), are not noticeable in the exposed individuals but only in the subsequent generations (Nilsson et al., 2018; Shaw et al., 2017). These effects might be multigenerational or transgenerational; the main difference between both is whether the brood was directly exposed (multigenerational) or not directly exposed (transgenerational) to a certain stimulus. In the case of daphnids, this means that if a F0 daphnid is subjected to a stimulus, then the brood inside that female that will give rise to the F1 was also exposed. Because the unborn neonates already contain germinative cells of the next generation, the F2 was also exposed to the contaminants and thus this generation can also present multigenerational effects; only F3 and future generations, that were never exposed, correspond to transgenerational effects (Jeremias et al., 2018; Xin et al., 2015). Figure 8 illustrates the differences between multigenerational and transgenerational in *D. magna*.

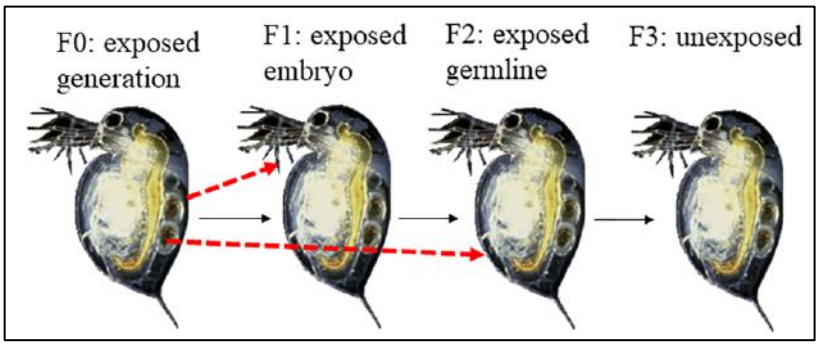

Figure 8: Schematic representation of how tests with daphnids are considered multigenerational (F1 and F2) and transgenerational (F3). Adapted from Jeremias et al. (2018).

D. magna fills out the so important requirements for its potential use in transgenerational tests because: 1) given the long-term nature of transgenerational tests, using an organism with a short life cycle such as *D. magna* drastically reduces the experimental time and 2) the interference of genetic factors will be theoretically minimal due to the fact that *D. magna* broods are clones of their respective progenitor. However, one has to realize that transgenerational tests performed with these organisms will act only as a starting-point in the toxicity assessment of a given contaminant but can very informative for upcoming transgenerational tests in more complex animals and, ultimately in humans (Martins and Guilhermino, 2018; Pembrey et al., 2014).

Chapter II – Objectives

Oxidative stress is a conserved widespread toxic mechanism; therefore, aquatic biota may respond to stress insults by triggering additional oxidative stress, and thus may amplify oxidative stress throughout the environment as a contagious disease.

The main objective of this thesis is to evaluate the chronic toxicity of oxidative stress triggers, namely isoprostanes and sodium hypochlorite, towards the test organism *Daphnia magna*.

Furthermore, we also aim to understand if isoprostanes can be considered as an example of circular toxicity. Isoprostanes are a product of lipid peroxidation, which by itself is a consequence of oxidative stress, and since isoprostanes are mainly excreted through human urine, they will be present in sewage systems and eventually reach wastewater treatment plants. Ultimately, isoprostanes will make their way to the aquatic environment where exposed species can interact with them, triggering a response that can result in the excretion of more isoprostanes to the environment.

In order to achieve these objectives, acute, chronic, multi and trans-generational tests with the crustacean *D. magna* were performed. Additionally, new miniaturised protocols were validated for acute tests in order to improve resource efficiency.

Chapter III – Methods

3.1. Test organism

Stock cultures of *D. magna* were routinely maintained in the lab at constant temperature of 20°C with a photoperiod of 16h of light and 8h of darkness, with the original clones coming from a well-established lineage from the applEE R&D lab - Applied Ecology and Ecotoxicology Laboratory, Department of Biology, University of Aveiro. Stocks of 20 daphnids were grown in a 800mL beaker with reconstituted ASTM fresh water medium (see Annex I), with the medium being renewed every other day.

Cultures were fed with the algae *Raphidocellis subcapitata* (cultured in the laboratory in Woods Hole MBL Medium, see Annex II) at a concentration of $3.0x10^5$ cells/mL/daphnia every day and supplemented with 4.8mL of diluted algae extract (Annex III) every other day.

The daphnids were maintained in the lab until their sixth brood (N6) and new cultures were initiated with neonates from the third to the sixth brood (N3-N6). Time for the first brood and date of birth for the subsequent broods were always registered.

3.2. Test procedures

3.2.1. Test solutions

3.2.1.1. Potassium dichromate

A stock solution of 100 $mg.L^{-1}$ of potassium dichromate (AnalaR NORMAPUR®, purity ≥99.8%) was prepared by dissolving 10mg of $K_2Cr_2O_7$ in 100mL of ASTM moderately hard water. Once prepared, the solution was stored at 4°C for 7 days as recommended by Baumann et al. (2014). From this stock solution, a new stock of 10 $mg.L^{-1}$ solution was prepared by adding 25mL of the first stock to 225mL of ASTM moderately hard water for a final volume of 250mL. Once prepared, the solution was also stored at 4°C during 7 days. This 10 $mg.L^{-1}$ stock solution, was used to prepare the potassium dichromate test solutions. Six different concentrations (0, 0.2, 0.4, 0.8, 1.6 and 3.2 $mg.L^{-1}$) were prepared in quadruplicate, with 5 daphnids per replicate in a 6 well plate.

These concentrations were prepared in increasing order of concentration using a volumetric flask of 50mL. The preparation of the control solution (ASTM moderately hard water) consisted in a direct pipetting of 10mL of ASTM moderately hard water to its respective wells in all replicates (plates).

When preparing the different solutions of $K_2Cr_2O_7$, a volumetric flask of 50mL was filled to half with ASTM moderately hard water. Then, the volume needed to obtain the respective $K_2Cr_2O_7$ concentrations was pipetted to the volumetric flask (1, 2, 4, 8 and 16 mL, respectively) and,

after that, the volumetric flask was filled to its volume of 50mL again with ASTM moderately hard water. Once the solution was prepared, its content was poured into a 100mL glass beaker and 10mL of the solution test prepared was pipetted to its respective well, being this repeated for the respective four replicates. This procedure was then repeated for each of the different concentrations of the test solution. Finally, any remaining potassium dichromate solutions were discarded to an appropriate container (metal waste) and all glass material used was placed in an acid bath of 10% HNO_3.

3.2.1.2. Sodium hypochlorite

A stock solution of 1000 $mg.L^{-1}$ of sodium hypochlorite was prepared to a final volume of 50mL from a commercial sodium hypochlorite solution (Feritex - Produtos de Conservação e Limpeza, Lda) of 153 $g.L^{-1}$, in which 327µL were added to Milli-Q water to obtain the desired concentration. Afterwards, 100 mL a new stock solution [NaClO]=10 $mg.L^{-1}$, was prepared by adding 1mL of the 1000 $mg.L^{-1}$ stock solution to 99mL of ASTM hard water. Finally, from this 10 $mg.L^{-1}$ stock solution the different concentrations of the test solutions were prepared.

These solutions were prepared in increasing order of concentration, using a volumetric flask of 50mL. The preparation of the control solution (ASTM hard water) consisted in a direct pipetting of 10mL of ASTM hard water to its respective wells in all replicates (plates). When preparing the different solutions of NaClO, a volumetric flask of 50mL was filled to half with ASTM hard water. Then, the volume needed to obtain the respective NaClO concentrations was pipetted to the volumetric flask (range finding test – 0, 0.5, 1, 2, 4 and 8 mL, respectively; final test – 0, 2, 2.5, 3, 3.5 and 4 mL, respectively) and, after that, the volumetric flask was filled to its volume of 50mL again with ASTM hard water. Once the solution was prepared, its content was poured into a 100mL glass beaker and 10mL of the solution test prepared was pipetted to its respective well, being this repeated for the respective four replicates. This procedure was then repeated for each of the different concentrations of the test solution.

3.2.1.3. Isoprostanes

At first, 1mg of 8-iso prostaglandin $F_{2\alpha}$ (Cayman Chemical Company, purity ≥99%) was dissolved in a 10mL volumetric flask with Milli-Q water in order to prepare a stock solution of 0.1 $mg.mL^{-1}$ = 100 $mg.L^{-1}$). This stock solution was then separated in 10 aliquots of 1mL in eppendorf's that were stored at -20°C. From this initial solution, a stock of 200 $\mu g.L^{-1}$ was prepared by pipetting 20µL of the first stock solution to a 10mL volumetric flask with Milli-Q water. Finally, a 2 $\mu g.L^{-1}$ = 2000 ng. L^{-1} stock was prepared by pipetting 1mL of the former stock to a 100mL volumetric flask with Milli-Q water.

For this test, six different concentrations (0, 20, 200, 400, 800 and 1600 $ng.L^{-1}$) were used. These concentrations were prepared in increasing order of concentration using a volumetric flask of 50mL. The preparation of the control solution (ASTM hard water) consisted in a direct pipetting of 10mL of ASTM hard water to its respective wells in all plates. When preparing the

different solutions, a volumetric flask of 50mL was filled with a little of ASTM hard water. Then, the volume needed to obtain the respective concentrations was pipetted to the volumetric flask (0.5, 5, 10, 20 and 40 mL, respectively) and, after that, the volumetric flask was filled to its volume of 50mL again with ASTM hard water. Once the solution was prepared, its content was poured into a 100mL glass beaker and 10mL of the solution test prepared was pipetted to its respective well, being this repeated for the respective five replicates. This procedure was then repeated for each of the different concentrations of the test solution.

3.2.2. Acute toxicity tests

Acute toxicity tests were performed according to the OECD Guideline 202 (OECD/OCDE, 2004). For the determination of EC_{50} at 24h and 48h, neonates with less than 24h from healthy stocks and from a progeny between N3-N6 were used and the tests that were carried out at a constant temperature (20±2°C) with a photoperiod of 16h of light and 8h of darkness and without feeding.

Test were considered valid when mortality/immobilization in the control group didn't exceed 10%, no signs of stress or disease (discoloured daphnia) or odd behaviour (neonates trapped at the surface) occurred (OECD/OCDE, 2004).

According to the OECD guideline, acute toxicity tests should comprise a minimum of 20 daphnids properly divided between four replicates with each one having five daphnids in it, both in the control group and the test groups. For the test groups a minimum five test concentrations preferably arranged in geometric series should be tested. For each daphnid, a minimum of 2mL of volume should be provided, which translates to a minimal of 10mL for the five daphnids in each test group in each replica (OECD/OCDE, 2004). Generally, acute toxicity tests are performed in beakers with 50ml of test solution and 5 daphnids in each beaker.

3.2.2.1. Miniaturization

As previously mentioned, the OECD recommends at least 2ml for each animal. Thus, it's possible to use a "minimal volume", as established by the OCDE Guideline 202 without compromising the results. Besides, the miniaturization offers several advantages: (1) it reduces the number of animals, (2) uses smaller volumes of the test solutions (particularly important when testing toxic or expensive chemical solutions), (3) requires less space in the laboratory and (4) it allows to save time when preparing the experimental setup (Grintzalis et al., 2017). For aforementioned reasons, all acute toxicity tests performed throughout this thesis used this miniaturization approach.

The protocol was based on the one suggested by Grintzalis et al. (2017), with 6 well plates. To each well, 10 ml test solutions were added followed by the addition of 5 daphnids. For each concentration, 5 replicates were performed, thus each plate was considered an independent experiment. For each test, at least 4 independent experiments were performed.

3.2.3. Chronic toxicity tests

D. magna reproduction test were performed according to the OECD Guideline 211 (OECD/OCDE, 2012) to determine the reproductive output of parent daphnids exposed to different concentrations of a given compound for the time duration of 21 days. In order to a chronic test to be valid, 1) mortality in the control group cannot exceed 20% and 2) the mean of neonates from the control daphnids at the end of the test should be ≥60 (OECD/OCDE, 2012).

As in acute tests, neonates with less than 24h from healthy stocks and from a progeny between N3-N6 were used for tests carried out at a constant temperature of 20°C with a photoperiod of 16h of light and 8h of darkness. However, unlike acute tests, feeding and medium renewal is important and necessary: medium renewal – ASTM hard water, complemented with diluted algae extract is performed every other day and feeding is performed daily with concentrated algae (*R. subcapitata*) in order to avoid possible dilution of the test concentrations. Also, after moulting, a photograph was taken to the antenna region, in order to measure its exopodite.

The test procedure can be described as follows: on day 0 of the test, neonates with less than 24h from a N5 brood were introduced to the already prepared test vessels. From this point on, they were feed every day and medium renewal was performed at test days 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20. During the test, parent daphnids were transferred with the use of a Pasteur's plastic pipette with the tip cut, therefore allowing the passage of bigger adult-sized daphnia.

The endpoints addressed in this test were reproductive success, time for the first brood and following broods, number of immobilized neonates, aborted eggs and exopodite length (used to determine daphnia's length).

According to the guideline, each test concentration should have 10 daphnids (replicates) with five tests concentrations and one control group, each one maintained in its own test vessel, in a minimal volume of 50mL (OECD/OCDE, 2012). However, aiming at reducing the number of animals and contaminants used while facilitating logistics, the chronic toxicity tests in this thesis were performed only with 5 replicates per group. This reduction is particularly important because: 1) one of the goals of this thesis is to introduce test miniaturization in every test made and if miniaturization in this particular test proves that it doesn't compromise endpoints statistical significance, it will allow to reduce resources (medium, algae and test compound) as well time and space in the laboratory and 2) allows to perform more easily and at the same time, subsequent multigenerational tests (see section 3.2.4 for more details).

3.2.4. Multigenerational and transgenerational tests

Contrarily to acute and chronic tests, there are no OCDE guidelines for multigenerational and transgenerational tests. However, these tests can be seen as an extension of chronic tests since both of these are quite similar in terms of execution: neonates from an ongoing chronic test and from a N3 brood or above are used to launch the respective multigenerational test. The

way this is done is to take a single neonate with less than 24h from all the replicates of the chronic test and transfer to a test vessel with only the ASTM medium.

Conditions are all maintained the same as in the chronic tests constant temperature of 20°C with a photoperiod of 16h of light and 8h of darkness with 50mL ASTM Hard medium, with algae feeding being made every day and supplementation with diluted algae extract being made every other day (coincident with medium renewal days). Also, because all test vessels don't possess any test substance, it isn't mandatory to use algae with high absorvances. Multigenerational tests are performed with the neonates of the progenitors of the chronic tests (generation F1) and with the neonates of generation F1 (F2 generation) whereas transgenerational tests are performed with the neonates of F2 generation (which gives generation F3).

3.3. *Test validation – potassium dichromate test*

In order to evaluate the possible toxic effects of any contaminants in *D. magna*, it's necessary to evaluate the fitness of the test organisms through an acute toxicity test with a reference substance. According to OECD Guideline 202 (OECD/OCDE, 2004), using the reference substance potassium dichromate ($K_2Cr_2O_7$), the concentration estimated to immobilise 50% of neonates after 24h (EC_{50}-24h) should be within the range of 0.6-2.1 $mg.L^{-1}$. This validation test should be performed at least twice a year.

Hence, following the recommendation of the OECD Guideline 202, the test was performed every other month with neonates with less than 24h from the N3-N6 brood. The tests were performed in quadruplicate, with 5 daphnids per replicate in a 6 well plate, following the procedure described in the previous section. The results of all potassium dichromate tests performed between July 2017 and September 2018 are summarized in section 4.1. of the Results chapter.

3.4. Sodium hypochlorite tests

3.4.1. NaClO acute toxicity tests

NaClO was used due to the presence of hypochlorite anion, a reactive species. The acute toxicity test was performed following the protocol described in section 3.2.2. In order to calculate the EC_{50}(48h), which determines the concentrations to be used in the subsequent chronic tests (section 3.2.3), two tests were performed for a more accurate determination of toxicity. Both tests were performed in the exact same way, only differing in the test concentrations used (first test – 0, 0.1, 0.2, 0.4, 0.8 and 1.6 $mg.L^{-1}$; second test – 0, 0.4, 0.5, 0.6, 0.7 and 0.8 $mg.L^{-1}$) and in the neonates brood used (first test – N3 brood; second test – N4 brood). In both tests, the respective six different concentrations were used in quadruplicate, with 5 daphnids per replicate in a 6 well plate. The number of immobilized daphnids were registered at 24 and 48h and the results of both tests are described in section 4.2.1 of the Results chapter.

3.4.2. NaClO chronic toxicity tests

The chronic toxicity test was performed taking into account the EC_{50}(48h) obtained in the acute toxicity tests, in order to guarantee that the highest test concentrations of the chronic test wouldn't induce death on the respective daphnids. Therefore, the concentrations used in this test were 0, 0.045, 0.09, 0.18, 0.36 and 0.72 $mg.L^{-1}$. These test concentrations were prepared freshly before medium renewal from the stock solution of [NaClO] = 10 $mg.L^{-1}$. The test procedure is described in section 3.2.3 and the results obtained can be seen in section 4.2.2.

3.4.3. NaClO multigenerational and transgenerational tests

In order to address multigenerational effects, neonates born in the chronic test are used and to launch transgenerational tests, neonates born in multigenerational tests are used. To accomplish this, N3 neonates from the F0 generation were used for the F1 multigenerational test. From the N3 neonates born in the F1 multigenerational test, another multigenerational test (F2) was initiated and later, with the N3 brood of the F2 generation, the transgenerational test was launched. Both multigenerational and transgenerational tests, were performed similarly to what's described in section 3.2.4. Daphnids were feed every day, with ASTM Hard Water renewal (50mL) every other day, as well as addition of algae extract. Results obtained are present in section 4.2.3 and section 4.2.4, respectively. The endpoints for these tests were reproductive output, parent mortality, time for the first brood and following broods.

3.5. Isoprostanes tests

3.5.1. IsoPs acute toxicity tests

The acute toxicity test was performed as described in section 3.2.2. with 5 different concentrations tested in quadruplicate, with 5 daphnids per replicate in a 6 well plate. The number of immobilized daphnids were registered at 24 and 48h and the results of both tests are described in section 4.3.1.

Because the toxicity of IsoPs towards *D.magna* was, to this date and to our best knowledge, never performed, the range of concentrations tested (0.02, 0.2, 0.4, 0.8 and 1.6 $\mu g.L^{-1}$) included, in the lower range, the values reported in the environment and, in the higher range, concentrations three orders of magnitude higher than the environmental levels reported.

3.5.2. IsoPs chronic toxicity tests

The concentrations used for the chronic toxicity test were 0, 0.02, 0.2, 0.4, 0.8 and 1.6 $\mu g.L^{-1}$ and were prepared from the [8-iso prostaglandin F2α] = 200 $\mu g.L^{-1}$ stock solution preserved at -20ºC. For this, the six different concentrations were prepared every time medium renewal was performed. The test procedure is described in section 3.2.3.

3.5.3. IsoPs multigenerational tests

For IsoPs, only a F1 multigenerational test was performed. As with the NaClO multigenerational test (section 3.2.4), N3 brood neonates from the IsoPs chronic test (F0) were used. Maintenance was performed as described above: feeding every day, while ASTM Hard Water (50mL) was renewed every other day, alongside with addition of algae extract. Identically to NaClO multigenerational tests (section 3.4.3), the same endpoints were addressed.

3.6. Body length measurement

Body length estimations were performed by using an allometric relation between the exopodite length (EL) and body length (BL) in *D. magna* as suggested by (Pereira et al., 2004). For this, every time daphnids shedded after brood release, photos were taken to their molt. The pictures were taken in a Nikon SMZ 745T Model C-LEDS with an imaging source DFK 72AUC02 camera. All the pictures were taken in IC Capture 2.4 (video size and colour format of RGB24 (1280x960), 7.50 FPS, BMP file with depth of 24 bits) at 5x magnification. The pictures were taken to the antennae area so that the exopodite was centred and focused. The exopodite to be measured is depicted in Figure 9.

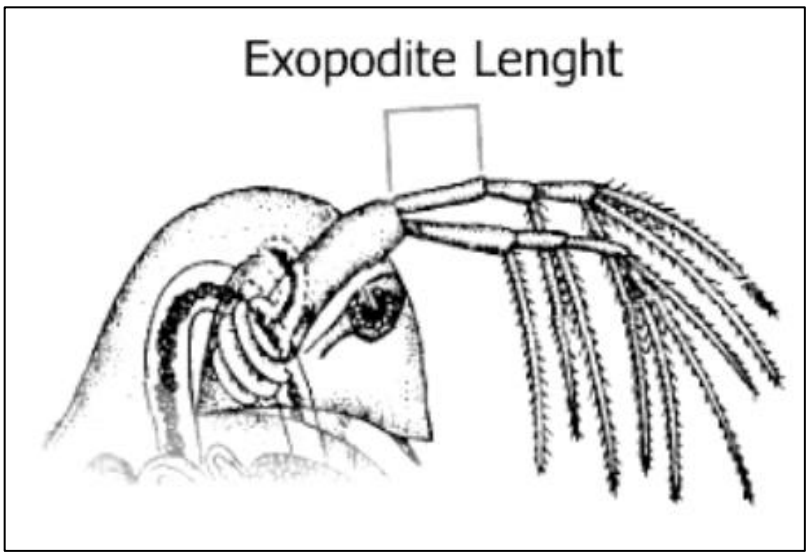

Figure 9: Representation of the exopodite in the second antennae, as well how to properly measure it. Adapted from (Mesquita, 2005).

After this, the software Axiovision SE64 Rel. 4.9.1 was used to measure the exopodites. Before this could be performed, a calibration was defined using a 0.5 mm microscope ruler slide with the magnifier set at 5x, with the calibration consisting in the measuring of 100 µm (=0.7704 µm/pixel in both xx and yy axis). Exopodite photos were then measured 3 times, with the need to define the previous calibration every time a new photo was measured.
Once all exopodites were measured, the mean obtained for each one was then used in the following equation (Pereira et al., 2004):

$$y = mx + b \Leftrightarrow y = 10.499x - 0.329 \quad (1)$$

where y = BL and x = EL.

The final measurements of daphnia's body length are available in sections 4.2.2.4 (NaClO) and 4.3.2.4 (IsoPs).

3.7. Statistical analysis

EC_{50}s determination in acute toxicity tests were performed with the software SigmaPlot 12.5. For this, the respective values of concentrations used and the immobilization registered at the end of the test were inputted in different columns, to represent the xx and yy axis, respectively. After this, an analysis using the option "regression wizard" was selected and after that, a report is generated considering the results. At this point, the R^2 and the standard error are analysed to see if the obtained model is statistically significant and, if so, the EC_{50} will correspond to the value given by the x0 variable.

Chapter IV – Results

4.1. Tests validation – potassium dichromate test

The validation of toxicity tests with *D.magna*, as stipulated in OECD/OCDE (2004), was performed with potassium dichromate ($K_2Cr_2O_7$). Table 3 describes the results for all $K_2Cr_2O_7$ tests performed between June 2017 to September 2018. The tests were performed using neonates from brood 3 to 6 and the EC_{50} values varied between 0.873 and 1.72 $mg.L^{-1}$. Such values are in agreement with the range reported by OECD (0.6-2.1 $mg.L^{-1}$) and thus the tests are valid.

Table 3 - Acute toxicity of *D. magna* for $K_2Cr_2O_7$ from June 2017 to September 2018.

Date	Brood	EC_{50} $mg.L^{-1}$	Mortality control (%)
29/06/2017	N4	0.873	0
28/07/2017	N6	1.66	0
16/11/2017	N5	1.71	0
22/12/2017	N6	1.72	0
11/04/2018	N5	1.6	0
08/06/2018	N5	1.72	0
23/09/2018	N3	1.68	0

4.2. NaClO

4.2.1 Acute toxicity

Acute toxicity tests were performed in order to determine the EC_{50} at 48h. At first, the following concentrations were tested: 0.0 (control), 0.1, 0.2, 0.4, 0.8, 1.6 (Table 4). The obtained EC50 was 0.6043 mg $NaClO.L^{-1}$.

Table 4 - Acute toxicity of *D. magna* for NaClO at both 24h and 48h.

	[NaClO] ($mg.L^{-1}$)					
Replica (N3)	**CTRL**	**0.1**	**0.2**	**0.4**	**0.8**	**1.6**
1	0	0	0	0	5	5
2	0	0	0	0	5	5
3	0	0	0	0	5	5
4	0	0	0	0	5	5

EC_{50}(24h) = EC_{50}(48h) = 0.6043 mg $NaClO.L^{-1}$

Since there was a gap of concentrations between the two concentrations were immobilization was registered, another acute toxicity test was performed taking into consideration that the middle concentration would had to match with the previous EC_{50}(48h) obtained. The concentrations used and the results obtained at 24h and 48h are depicted in Tables 5 and 6. The obtained EC_{50} for 24h was 0.780 $mg.L^{-1}$, whereas for 48h it was 0.7804 $mg.L^{-1}$.

Table 5 - Acute toxicity of *D. magna* for NaClO at 24h.

	[NaClO] (mg.L^{-1})					
Replica (N4)	**CTRL**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**
1	0	0	0	0	0	5
2	0	0	0	0	0	4
3	0	0	0	0	0	5
4	0	0	0	0	0	5

EC_{50}(24h) = 0.7800 mg NaClO.L^{-1}

Table 6 - Acute toxicity of *D. magna* for NaClO at 48h.

	[NaClO] (mg.L^{-1})					
Replica (N4)	**CTRL**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**
1	0	0	0	0	0	5
2	0	0	0	0	0	5
3	0	0	0	0	0	5
4	0	0	0	0	0	5

EC_{50}(48h) = 0.7804 mg NaClO.L^{-1}

4.2.2. Chronic test – F0

Chronic test concentrations were selected taking into consideration the EC_{50}(48h) obtained with the results depicted in Table 6. The criteria used was to select concentrations below the EC_{50}(48h) with a factor of 2 between them. The chronic test concentrations were: 0.00 (control), 0.045, 0.09, 0.18, 0.36 and 0.72 mg(NaClO).L^{-1}.

4.2.2.1. Time for first brood

Every daphnia from all the test groups involved in this test gave their first brood at the 8th day of the test.

4.2.2.2 Reproductive success

In the N1 brood of the chronic test, it's possible to observe two tendencies: the lower concentrations that gave similar number of neonates to the control group (0.045, 0.09 and 0.18 mg.L^{-1}) and the higher concentrations that gave fewer neonates (0.36 and 0.72 mg.L^{-1}). In the N2 brood, the lowest concentrations (0.045 and 0.09 mg.L^{-1}) registered the highest number of neonates, followed by the control group, with the remaining number of neonates decreasing as concentrations increased. In fact, from N2 onwards, the number of neonates in the highest concentrations (0.18, 0.36 and 0.72 mg.L^{-1}) decreased as the concentration increases. The N3 brood was similar to the previous brood, with the highest number of neonates being registered for the concentration of 0.045 mg.L^{-1} and the control group having similar number of neonates as the 0.09 mg.L^{-1} group. For the N4 brood, the control group and the lowest concentration group (0.045 mg.L^{-1}) registered identical number of neonates, with the 0.09 mg.L^{-1} concentration giving the highest number of neonates for this brood. In the last brood of this test (N5), the 0.09 mg.L^{-1} concentration registered once again the highest number of neonates

per brood, followed by the control group and with the lowest concentration (0.045 $mg.L^{-1}$) registering similar number of neonates as the 0.18 $mg.L^{-1}$ group.

Overall, the general tendency for all concentration in the different broods of this test was, as daphnid parents got older, the higher number of neonates were obtained, i.e. the bigger the size of their broods (Figure 10).

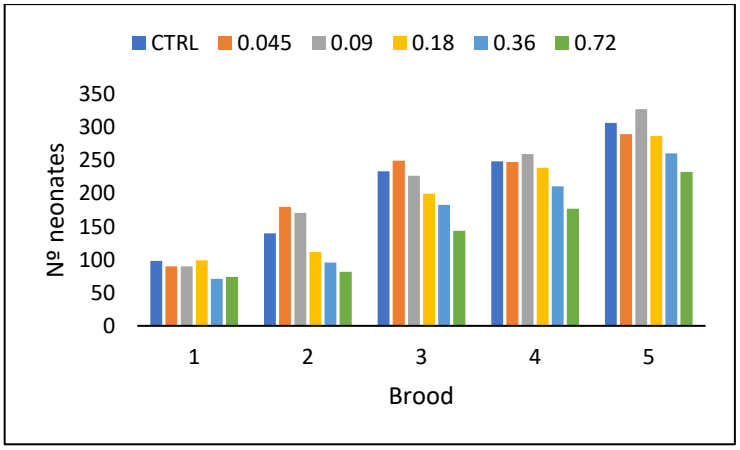

Figure 10: Number of neonates obtained between broods for each group in the chronic test. Each bar corresponds to the sum of the respective replicates in that brood.

The pattern of total number of neonates for the different concentrations in this chronic test is similar to what happened in the individual broods, with the control and lowest concentrations (0.045 and 0.09 $mg.L^{-1}$) giving rise to more neonates while the highest concentrations (0.18, 0.36 and 0.72 $mg.L^{-1}$) registered less neonates. Figure 11 summarizes this tendency, with the total number of neonates for each group corresponding to the sum of neonates obtained in every brood depicted in Figure 10.

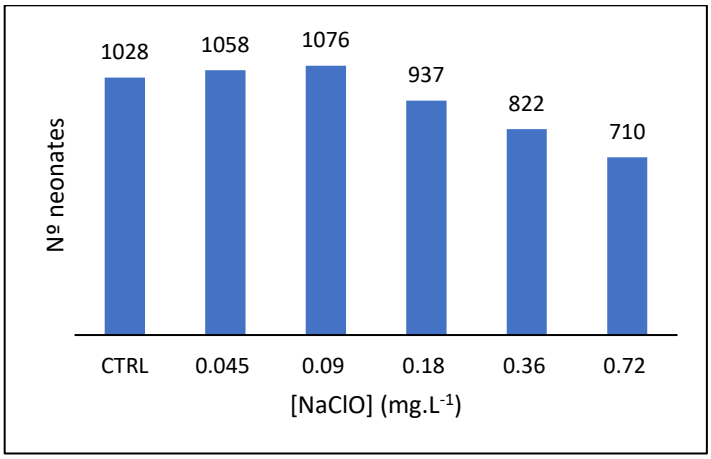

Figure 11: Total number of neonates obtained throughout the chronic test for each test concentration.

4.2.2.3 Immobilization

In the control group no immobilized neonates were found, while in the lowest concentrations (0.045 and 0.09 $mg.L^{-1}$) 2 and 1 immobilized neonate were recorded, respectively. From 0.18 $mg.L^{-1}$ onwards, the tendency observed was that the highest the concentration, the more immobilized neonates were recorded, as 0.18 $mg.L^{-1}$ recorded 5 immobilized neonates, 0.36 $mg.L^{-1}$ recorded 8 immobilized neonates and 0.72 $mg.L^{-1}$ recorded 9 immobilized neonates (Figure 12).

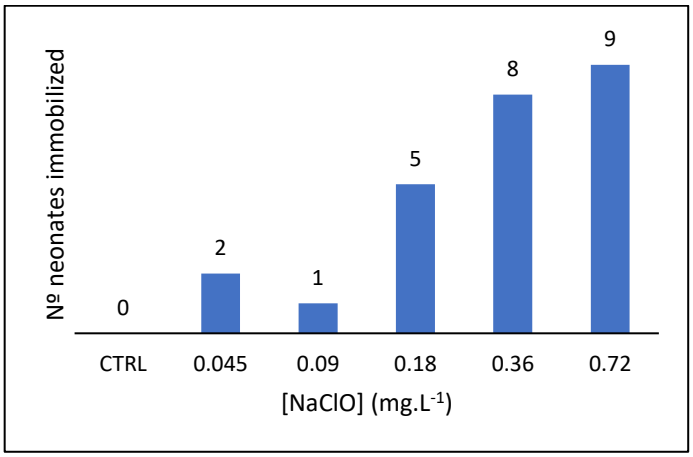

Figure 12: Total number of immobilized neonates in the chronic test in each test concentration.

4.2.2.4 Body length size

Daphnids body length was determined by measuring their exopodite in their molt after every brood by applying equation (1), as described in section 3.6. In the N1 brood, almost all concentrations registered the same body length except for 0.045 $mg.L^{-1}$ group and, to a less extent, 0.09 and 0.18 $mg.L^{-1}$. In the N2 brood, daphnids from the 0.045 and 0.09 $mg.L^{-1}$ groups presented a larger body length when compared to the remaining concentrations, with the control group being slightly larger than 0.18 $mg.L^{-1}$ and then 0.36 and 0.72 $mg.L^{-1}$. N3 and N4 were quite similar (larger sizes in low concentrations, smaller sizes in high concentrations), with the only exception being that, 0.18 $mg.L^{-1}$ group registered a larger body length in N3 while the control group registered its largest body length in the N4. At last, for the N5 brood, lowest concentrations and control groups registered larger body lengths (0.09 > 0.045 > CTRL) while highest concentrations register smaller body lengths (0.18 > 0.36 > 0.72).

Overall, although with different body lengths between the different test groups, all body lengths increased as the broods progressed (Figure 13).

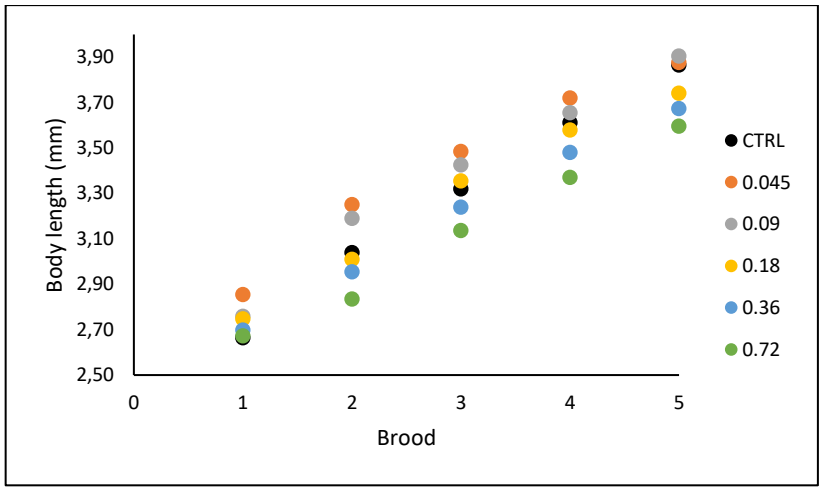

Figure 13: *D. magna* body length of the different test groups throughout their broods.

4.2.3 Multigenerational tests – F1 and F2

For multigenerational tests, contrarily to chronic ones, the tests were performed only in ASTM medium (with no added NaClO) since the objective of these tests is to analyse the potential effects in the exposed embryo (F1) and exposed germlines (F2). It is also important to stress that F1 multigenerational test used N3 neonates from the prior chronic F0 test and the F2 multigenerational test used N3 neonates from its F1 multigenerational predecessor test.

4.2.3.1 Time for first brood

In the multigenerational test with the F1 generation, all the test groups daphnids gave their first brood at the 8th day of the test. Regarding the F2 generation, all the test groups daphnids gave their first brood at the 9th day of the test.

4.2.3.2 Reproductive success

The N1 brood of the F1 multigenerational test registered the lowest number of neonates, raising from the F0-control group to the group F0-0.36, and then decreasing to reach its minimum in the group F0-072. For the N2 brood, the F0-control group presented the highest number of neonates and afterwards a decreasing tendency was observed (except for F0-0.045 which was similar to F0-0.36). In the N3 brood, the number of neonates in the F0-control group, F0-0.18 and F0-036 decreased, while F0-0.09 and F0-0.72 stayed almost the same and F0-0.045 raised. Regarding N4, all test concentrations presented lower number of neonates, with the F0-control group and F0-0.72 being the ones registering the highest numbers for this brood, followed by F0-0-09, F0-0.045 and, with almost the same value, F0-0.18 and F0-0.36. In the last brood (N5), the number of neonates in all test concentrations increased, with F0-control and F0-0.72 once again registering the highest numbers, followed by F0-0.045 and F0.036 (which also presented similar number of neonates), F0-0.09 and with F0-0.18 having the lowest number of neonates in N5 (Figure 14).

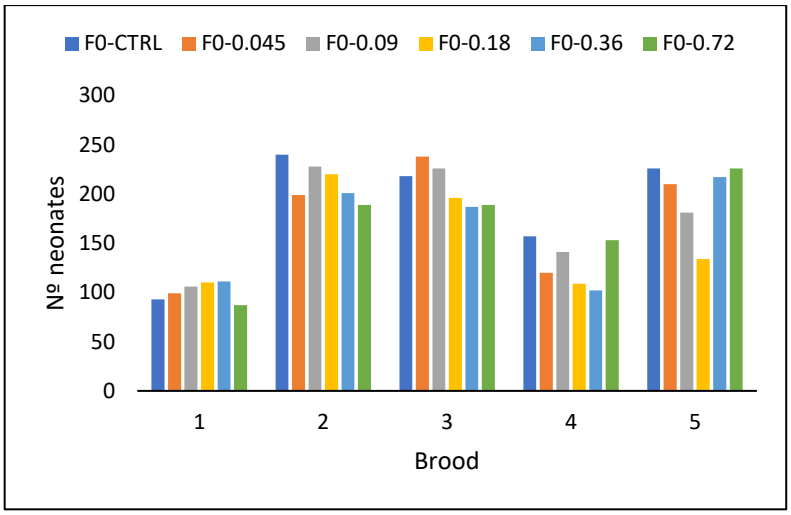

Figure 14: Number of neonates obtained between broods for each group in the multigenerational F1 test. Each bar corresponds to the sum of the respective replicates in that brood.

Observing the total number of neonates for the multigenerational F1 test, F0-control presents the highest number, being followed by F0-0.045 and F0-0.09, which have roughly the same value. Afterwards, appears F0-0.72 which is, in turn, followed by F0-0.36 and, distinct from all the tests groups referred above, comes F0-0.18, with the lowest number of neonates registered. Figure 15 summarizes this, with the total number of neonates for each group test corresponding to the sum of neonates obtained in every brood depicted in Figure 14.

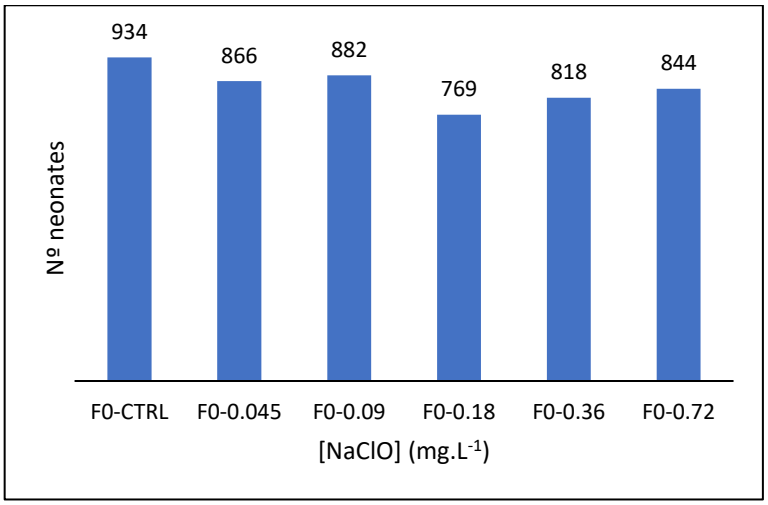

Figure 15: Total number of neonates obtained throughout the multigenerational F1 test for each concentration.

In the multigenerational F2 test, the N1 brood was the one where the lowest number of neonates were registered. In this brood, F0-0.045 was the test group with the highest number of neonates and F0-0-72 was the one with the lowest number. Regarding the remaining group tests, F0-CTRL and F0-0.36 presented similar number of neonates, as well as F0-0.09 and F0-0.18. For the N2 brood, two different groups can be discerned: F0-CTRL, F0-0.09 and F0-0.36 with the highest number of neonates, by this order, and F0-0.045, F0-0.18 and F0-0.72 with the lowest number, respectively. N3 brood presented F0-CTRL and F0-0.045 with the same number

of neonates while the remaining test groups followed a decreasing pattern from F0-0.09 (maximum value) to F0-0.72 (minimum value). Concerning N4, F0-0.045 and F0-0.09 had almost the same number of neonates, with the last one being the maximum for this brood. These groups are followed by F0-0.36, then by F0-CTRL and F0-0.18 (also more or less similar) and, once again, the lowest number of neonates being registered in F0-0.72. Lastly, all groups in N5, except for F0-0.18 and F0-0.72, presented more than 200 neonates (Figure 16).

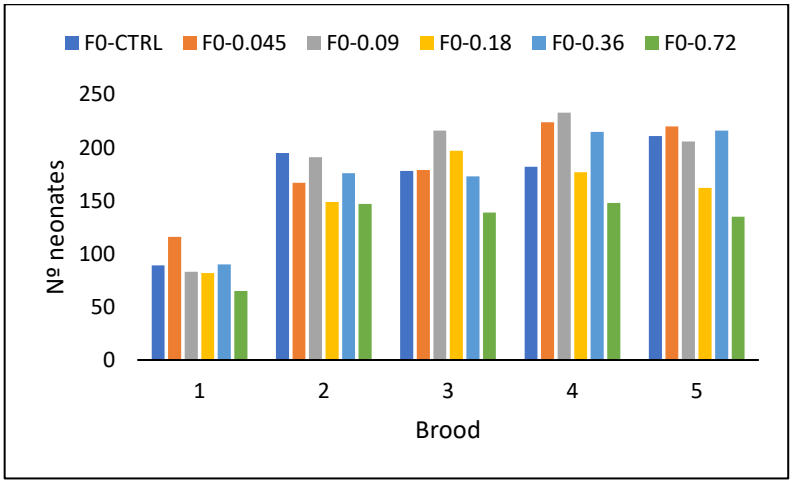

Figure 16: Number of neonates obtained between broods for each group in the multigenerational F2 test. Each bar corresponds to the sum of the respective replicates in that brood.

For the F2 multigenerational test, the total number of neonates was similar between F0-0.45 and F0-0.09 (group with the highest value) and between F0-CTRL and F0-0.36, followed by F0-0.18. The group with the lowest total number of neonates was F0-0.72, as it happened throughout all broods. Figure 17 summarizes these findings, with the total number of neonates for each group corresponding to the sum of neonates obtained in every brood depicted in Figure 16.

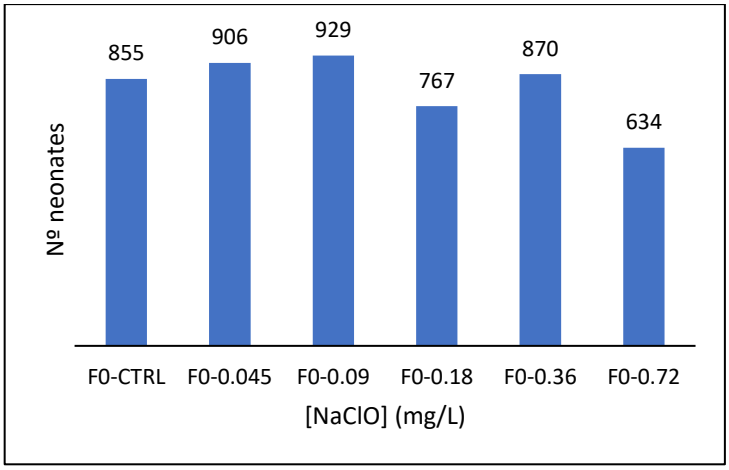

Figure 17: Total number of neonates obtained throughout the multigenerational F2 test for each concentration.

4.2.3.3 Immobilization

For both F1 and F2 multigenerational tests, no immobilized neonates were observed. However, in the particular case of the F2 multigenerational test, immobilization was observed in the replicates, i.e. mother daphnids. The recorded deaths were the following:

- Replicate 1 from the concentration F0-0.72 $mg.L^{-1}$, dead at the 7th day of the test (prior to giving N1 brood). The death of this daphnid would affect the following transgenerational test because there were no N3 neonates to use in that test.
- Replicate 4 from the concentration F0-0.18 $mg.L^{-1}$, dead at the 17th day of the test (between N3 and N4 brood). The death of this daphnid did not affect the following transgenerational test because it died after giving the N3 brood.

4.2.4 Transgenerational test – F3

For this transgenerational test, similarly to multigenerational tests, no NaClO was added to the medium as the objective of this test is to analyse its potential effects in unexposed daphnia.

4.2.4.1. Time for first brood

All daphnias from all the groups involved in the transgenerational F3 test gave their first brood at the 9th day of the test.

4.2.4.2 Reproductive success

The reproductive success in the transgenerational test on N1 had its highest number of neonates in the F0-0.18, being followed by F0-CTRL and F0-0.045. The groups F0-0.09 and F0-0.36 presented approximately the same number of neonates while F0-0.72 exhibited the lowest number of neonates in this brood. For the N2 brood, F0-0.18 was again the group with the maximum number of neonates, followed by F0-CTRL and F0-0.09. Regarding the remaining groups, F0-0.36 and F0-0.72 presented the same number of neonates and F0-0.045 was the one with lower neonates. In N3, once again F0-0.18 was the group with the highest number of neonates, with the other groups with high numbers being F0-0.09 and F0-CTRL. The groups F0-0.045 and F0-0.72 shared the same number of neonates and F0-0.018 was the one with less neonates in this brood. N4 brood presented test groups with high number of neonates (F0-0.09 > F0-0.36 > F0-0.18), F0-0.045 and F0-0.72 with almost the same number of neonates and with the F0-CTRL being the group with less neonates in this brood. Oppositely, to the previous brood, F0-CTRL was the group with the highest number of neonates, followed by F0-0.36. The groups F0-0.09 and F0-0.18 presented approximately the same number of neonates, followed by F0-0.72 and, lastly, the group test F0-0.045 was the one to give rise to less neonates in the N5 brood (Figure 18).

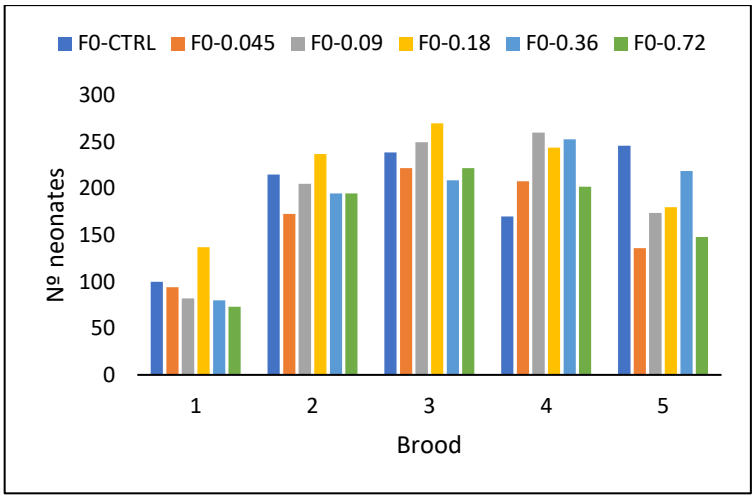

Figure 18: Number of neonates obtained between broods for each group in the transgenerational F3 test. Each bar corresponds to the sum of the respective replicates in that brood.

The results from the total number of neonates for the transgenerational F3 test showed that the group test F0-0.18 had the highest number, being followed by F0-CTRL and F0-0.09 with identical number of neonates. These groups are then followed by F0-0.36 and, finally, both F0-0.72 and F0-0.045, with this last one recording the lowest number of total neonates. Figure 19 summarizes these findings, with the total number of neonates for each group corresponding to the sum of neonates obtained in every brood depicted in Figure 18.

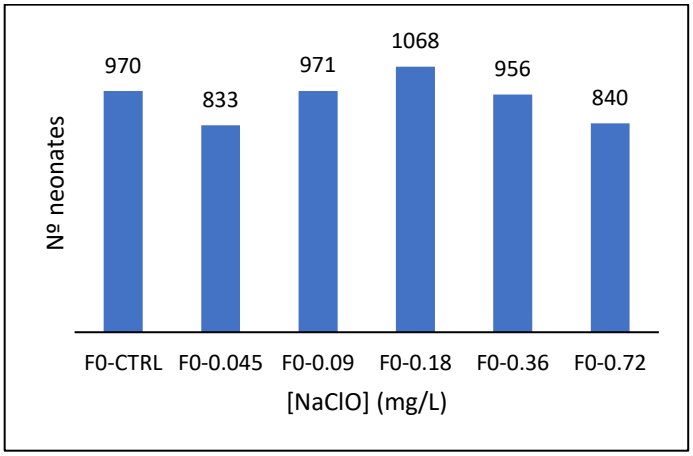

Figure 19: Total number of neonates obtained throughout the transgenerational F3 test for each group.

4.2.4.3 Immobilization

In this test, immobilization was recorded in the replicates, i.e. mother daphnids. The recorded deaths were the following:

- Replicate 3 from the concentration F0-0.045 mg.L^{-1}, dead at the 11th day of the test (between N1 and N2 brood).
- Replicate 2 from the concentration F0-0.18 mg.L^{-1}, dead at the 18th day of the test (between N4 and N5 brood).

Also, because replicate 1 from the concentration F0-0.72 mg.L^{-1} of the multigenerational F2 test died before giving any brood, this test only had 4 replicates for the concentration of F0-0.72 mg.L^{-1}.

4.3. IsoPs – 8-iso Prostaglandin $F_{2\alpha}$

4.3.1 Acute toxicity tests

In order to access the adequate test concentrations of IsoPs (in this case, 8-iso-$PGF_{2\alpha}$) to be used in the chronic test with *D. magna,* acute toxicity tests were performed to determine the EC_{50}(48h). The criteria used for selecting the concentrations were: the lowest concentration of IsoPs should match the ones being currently reported in the environment (20 ng.L^{-1} – section 5.3.1, Figure 41) and the following concentrations to be one fold higher (200 ng.L^{-1}). Hence, the following concentrations were tested: 0.00, 0.02, 0.2, 0.4, 0.8 and 1.6 ug.L^{-1}. Because no immobilization was observed, in a second test we further increased the levels of IsoPs: 0, 2, 4, 8, 16 and 32 ug.L^{-1}. No immobilization was recorded for these unrealistic concentrations. Therefore, the EC_{50}(48h) for 8-iso-$PGF_{2\alpha}$ in *D. magna* wasn't determined and the criteria used to select the chronic test concentrations was different from the one employed in the chronic test performed with NaClO (section 4.2.2)

4.3.2 Chronic test

Contrarily to the chronic test using NaClO, no EC_{50}(48h) was determined for 8-iso-$PGF_{2\alpha}$ in *D. magna*. The reason why no more acute toxicity tests were made with even higher concentrations was due to the fact, in our view, it was more important to use concentrations that match the current reality of IsoPs levels in the environment (since toxicity effects of 8-iso-$PGF_{2\alpha}$ were never studied) than to be able to the determine an EC_{50}(48h) that would make us use chronic concentrations that are not realistic. For this reason, the first set of concentrations used in the acute toxicity test were used in this chronic test, namely 0.00 (control), 0.02, 0.2, 0.4, 0.8 and 1.6 ug.L^{-1}.

4.3.2.1. Time for first brood

Every daphnia from all the test groups involved in this test gave their first brood at the 9th day of the test.

4.3.2.2 Reproductive success

The N1 brood of this chronic test, as with other previously tests, was the brood which presented lower number of neonates for all concentrations, where the control group, 0.2 and 0.4 µg.L^{-1} had the highest numbers while 0.02, 0.8 and 1.6 µg.L^{-1} had the lowest numbers of neonates. For the N2 brood, 0.2 µg.L^{-1} group had the highest number of neonates, followed by 0.4 µg.L^{-1}. All of the remaining groups (except the control, the group with less neonates) presented similar number of neonates for this brood. The N3 brood was characterized by a rise in the number of neonates as the concentration was also higher; however, this occurred only between the control

and 0.4 µg.L^{-1}, as afterwards, as the concentration increased, the number of neonates started to decrease but the minimum value was not recorded here. By this, 0.4 µg.L^{-1} concentration registered the highest number of neonates while the lowest was registered in the control group. In N4, the 0.4 µg.L^{-1} concentration group registered again the highest number of neonates, with almost all the remaining groups exhibiting the same number. The only exception to this was 0.72 µg.L^{-1} that, although with almost the same numbers as the other groups, presented the lowest number of neonates for N4. In the last brood, the control group, 0.02, 0.2 and 0.4 µg.L^{-1} all presented roughly the same number, with once again 0.4 µg.L^{-1} registering the highest number of neonates, while 0.8 and 1.6 µg.L^{-1} presenting the lowest number of neonates in the N5 brood (Figure 20).

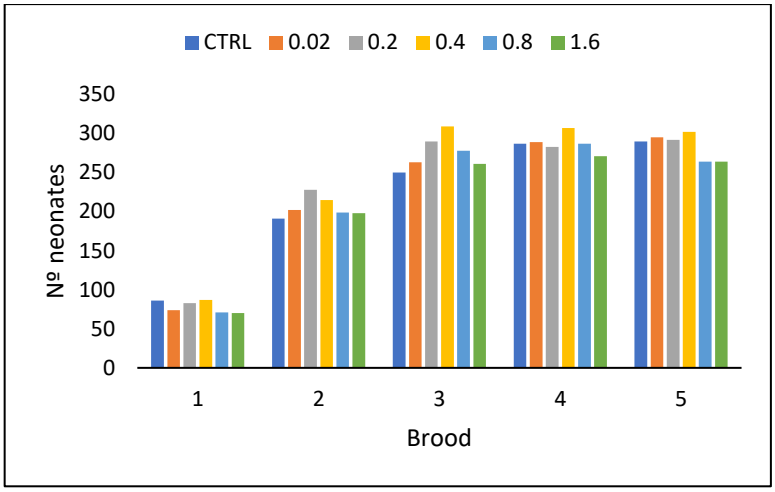

Figure 20: Number of neonates obtained between broods for each group in the chronic test. Each bar corresponds to the sum of the respective replicates in that brood.

As far as the total number of neonates goes, the tendency was that as the concentration of 8-iso-$PGF_{2\alpha}$ increased, the number of total neonates also increased for the concentration groups between the control group and the 0.4 µg.L^{-1} group. In fact, it was this last group that registered the highest number of neonates in the chronic test. For the remaining groups, 0.8 and 1.6 µg.L^{-1} were the ones with the lowest number of total neonates, with this last one being in fact the concentration group with the lowest number of neonates. Figure 21 summarizes these findings, with the total number of neonates for each group corresponding to the sum of neonates obtained in every brood depicted in Figure 20.

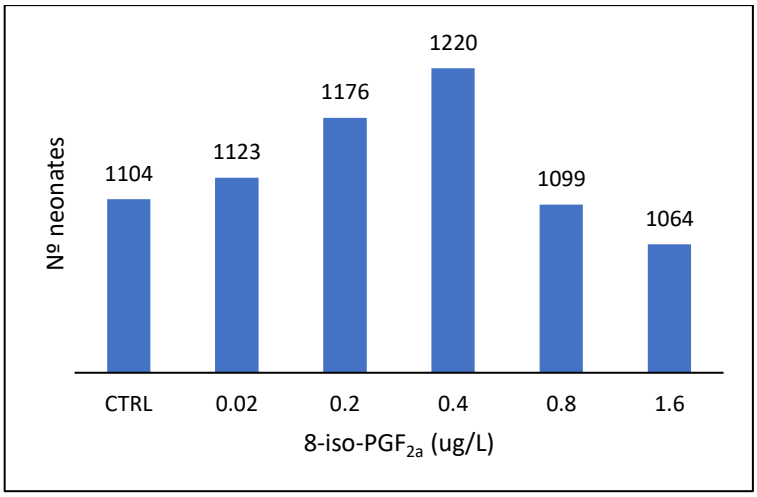

Figure 21: Total number of neonates obtained throughout the chronic test for each concentration.

4.3.2.3 Immobilization

No immobilization was recorded throughout the course of this chronic test, neither for neonates nor for daphnia replicates.

4.3.2.4 Body length size

Identically to what was performed in section 4.2.2.4, daphnids body length was determined by measuring their exopodite in their molt after every brood by applying equation (1), as described in section 3.6. In their first brood, body lengths were similar but is possible to see that the control group was the one with bigger daphnids and, in N2, the same happened but with this difference being more notorious. However, in the following broods (N3, N4 and N5), the body length of the daphnids in all groups when compared with the control group was practically the same (Figure 22).

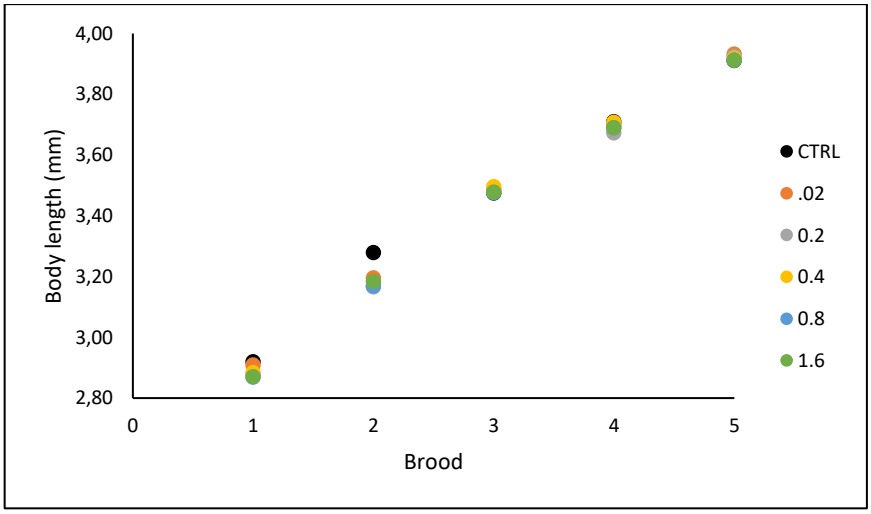

Figure 22: Body length of the various groups throughout their broods.

4.3.3 Multigenerational test – F1

As for the multigenerational tests with NaClO, no 8-iso-$PGF_{2\alpha}$ was added to the medium since the objective was to analyse the potential effects in unexposed embryos. Thus, this multigenerational F1 test used N3 brood neonates from the previous chronic F0 test.

4.3.3.1. Time for first brood

All daphnids from all the groups involved in this test gave their first brood at the 8th day of the test.

4.3.3.2 Reproductive output and success

In the N1 brood, although with the highest number of neonates, the test group F0-0.2 had similar results to the F0-CTRL, F0-0.02 and F0-0.4, while both F0-0.8 and F0-1.6 presented less neonates. In N2, both F0-CTRL and F0-0.2 presented similar higher number of neonates, with the remaining groups also giving practically the same number but with less neonates. In N3, F0-0.4 registered the highest number of neonates, followed by F0-0.02 and with the remaining groups sharing the same values but with F0-0.8 and F0-F0-1.6 registering the lowest ones. For N4, F0-0.02 presented the highest number, followed by both F0-CTRL and F0-0.2. For the other test groups, F0-0.4 and F0-1.6 also presented identical numbers, with the lowest number of neonates belonging to F0-0.8 group test. In the last brood it is important to note that all test groups had lower number of neonates compared to N4. F0-0.8 presented the highest value, followed by F0-1.6 and F0-CTRL. The other remaining groups presented the lowest number of neonates followed by F0-0.4, F0-0.02 and F0-0.2 (Figure 23).

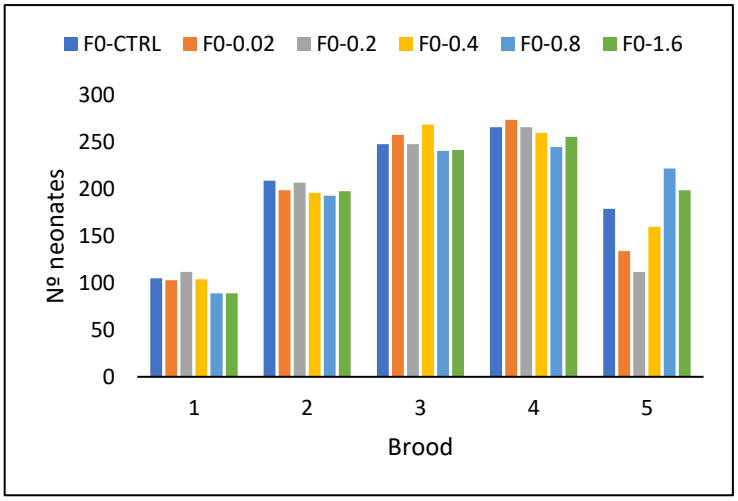

Figure 23: Number of neonates obtained between broods for each group in the multigenerational F1 test. Each bar corresponds to the sum of the respective replicates in that brood.

Overall, the F0-CTRL group had the highest total number of neonates in the test, followed by F0-0.4, F0-0.8 and F0-1.6 that shared similar numbers. The groups F0-0.02 and F0-0.2 presented the lowest number of neonates, with F0-0.2 exhibiting the lowest of all. Figure 24 summarizes these findings, with the total number of neonates for each group corresponding to the sum of neonates obtained in every brood depicted in Figure 23.

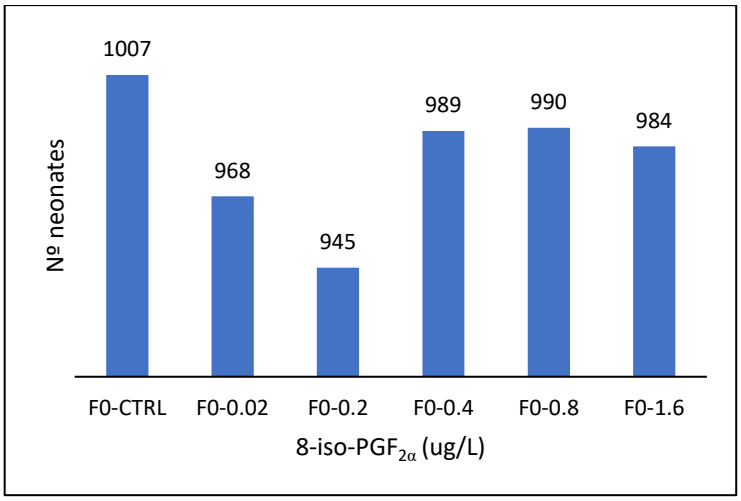

Figure 24: Total number of neonates obtained throughout the multigenerational F1 test for each group.

4.3.3.3 Immobilization

In this test, immobilization was recorded in the replicates, i.e. mother daphnids. The recorded immobilization was the following:

- Replicate 1 from the concentration F0-0.2 mg.L^{-1}, dead at the 20th day of the test (between N4 and N5 brood).

Chapter V – Discussion

5.1. Lethal and sublethal effects of NaClO towards *D. magna*

Although NaClO is extensively used and can easily contaminate aquatic environments, only acute toxicity studies are available. As such, not only a gap of information exists regarding its chronic effects, as well as the possible consequent multigenerational and transgenerational ones. Thus, and considering that hypochlorite is a reactive species that can induce oxidative stress, NaClO was used and its impact in the reproductive success of different generations of daphnids was studied.

5.1.1 Chronic test

From Figure 11 it is clearly noticeable that hypochlorite affected the reproductive success. However, this effect can be seen as positive for the lowest concentrations (0.045 and 0.09 $mg.L^{-1}$) and negative for the highest concentrations (0.18, 0.36 and 0.72 $mg.L^{-1}$), when compared to the number of neonates obtained in the control group. Also, when looking to the number of neonates per brood, although it increases for every concentration, the same pattern exists: lower concentrations sometimes have higher number of neonates but higher concentrations have fewer neonates, when compared to the control group. For this, one can say that chronic exposure to low hypochlorite concentrations can in fact be beneficial to daphnids and may be a consequence of a hermetic effect. As for higher hypochlorite concentrations, although not enough to induce immobilization, it affects the reproductive success in a way that parent daphnids give smaller broods.

In this test, neonates immobilization was also recorded (Figure 12). Although using NaClO concentrations below the $EC_{50}(48)$, some neonates didn't survive, particularly those from the highest concentrations. In fact, the highest the concentration the more immobilized daphnids were recorded. Finally, similarly to what happened with the reproductive success, not only the daphnids body length was influenced by NaClO concentrations, but also positively influenced by lower concentrations and negatively influenced by higher ones (Figure 13). Looking with more detail and comparing with the group control, all daphnids exposed to lower concentrations always were bigger while daphnids exposed to higher concentrations (particularly in concentrations 0.36 and 0.72 $mg.L^{-1}$) were always smaller. Considering that bigger body lengths can be synonymous to healthier daphnids and taking into account what was previously said, in a chronic context hypochlorite effects will depend on its concentration.

5.1.2 Multigenerational tests

When looking to the F1 multigenerational test, it is clearly noticeable that neonates that came from the control group show the highest total number of neonates (Figure 15). However, for the rest of the groups, it is not possible to say that there is a relation between the number of

neonates and the concentrations from where these daphnids originated, as it occurred in the chronic test. Looking to their respective broods, although it was expected for the number of neonates to increase with the number of broods and, eventually, being more or less constant in the last broods (as it happened untill the N3 brood), this didn't happen for the remaining broods of this test. In fact, in all groups the number of neonates decreased in N4 just to raise again in N5. However, not every group increased to its expected number, being that only exclusive for the F0-control and F0-0.045 groups.

The other multigenerational test (F2) was similar to the F0 test when it comes to the total number of neonates (Figure 17): the groups that came from the lowest concentrations presented higher number of neonates when compared to F0-CTRL; the F0-0.18 group presented less neonates when compared to F0-CTRL and F0-0.36 registered higher number of neonates than F0-CTRL. The F0-0.72 group was an exception that recorded a lower number of neonates but this was due in part to the fact that one replicate from this group died before giving the neonates. When comparing the number of neonates per brood for each group, although all broods had a higher number of neonates than the N1 brood, the remaining broods presented an oscillating tendency.

For all this, when comparing both multigenerational tests, we can conclude that: 1) N1 broods were always the ones to present less number of neonates in every test group (as expected since, at this stage, the mother daphnia are still young adults) and 2) the total number of neonates registered in the test group F0-0.72 was always lower than the total number of neonates registered in the control group F0-CTRL. However, regarding this last point it can not be ignored that, in the F2 test only 4 replicates existed since one replicate died at an early stage of this test.

5.1.3 Transgenerational test

When it comes to the F3 transgenerational test, the previous analyse becomes more difficult to make since this test ended without 3 of its replicates: one because it died in the F2 before giving any N3 neonates for this test and two throughout the course of the F3 test (belonging to the F0-0.045 and F0-018). Considering this and comparing to the F0-control group (Figure 19), F0-0.045 and F0-0.072 had both the lowest total number of neonates, F0-0.36 had slightly less total number of neonates, F0-0.09 presented practically the same number and F0-0.18 had the highest total number of neonates. Regarding the broods (Figure 18), once again N1 presented the lowest numbers and, when comparing to the F0-control group, almost all groups had lower number of neonates from N1 to N3 (the only exception being F0-0.18 group in all these broods and F0-0.09 in N3), in N4 all of the test groups showed higher number of neonates and in N5 all of the groups showed lower number of neonates.

Given all that has been discussed, it is difficult to compare trends between the various generations, broods and test groups in all of the tests. Figures 25 to 28 describe how all three of these variables relate over the course of the four tests performed with NaClO.

5.1.4. Overall tendencies

5.1.4.1 Reproductive success

The analysis of Figure 25 allows to conclude that, relatively to their broods and comparing within the different generations: F0 increases throughout all broods, F1 increases till N2, stays steady to N3, decreases in N4 and rises again in N5, F2 increases slightly throughout all broods to N4, then slightly decreases in N5 and F3 increases until N3 then starts to decrease. Also, comparing between generations relatively to the different concentrations: F0 slightly rises from 0.000 to 0.09, then starts to decrease, F1 and F2 have a similar behaviour for all concentrations and F3 stays steady to 0.09, rises at 0.18, decreases at 0.36 and slightly increases at 0.72.

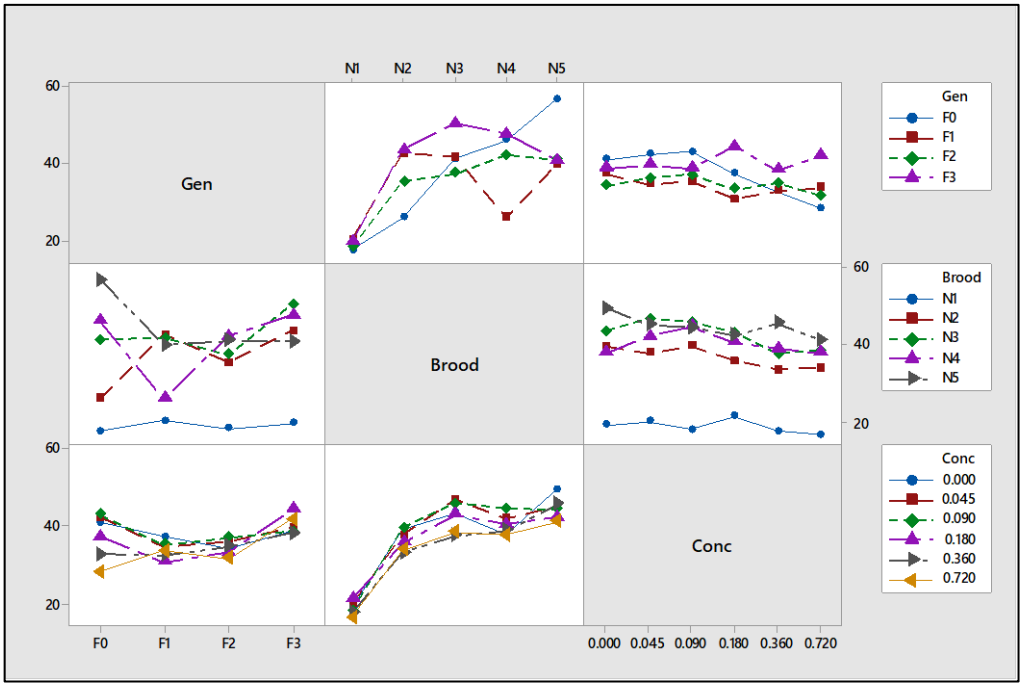

Figure 25: Summary of all the data regarding the reproductive success of daphnids exposed to NaClO. Gen: generation; Conc: concentration.

Figure 26 visually represents the main plots of all the three variables. In the different generations, F0 and F3 are the ones above the mean while F1 and F2 are under, with F3 having the highest while F1 was the lowest one. When it comes to the broods, N1 has the lowest mean of all, as well being the only one under the mean. From N2 to N3, there is an increase followed by a decrease in N4 that goes up again with N5, the brood with the highest mean. Finally, analysing the different groups, from the control group concentration to 0.09 (highest mean) the mean gradually rises, but from 0.18 to 0.72 the mean starts to fall till it reaches its minimum with 0.72.

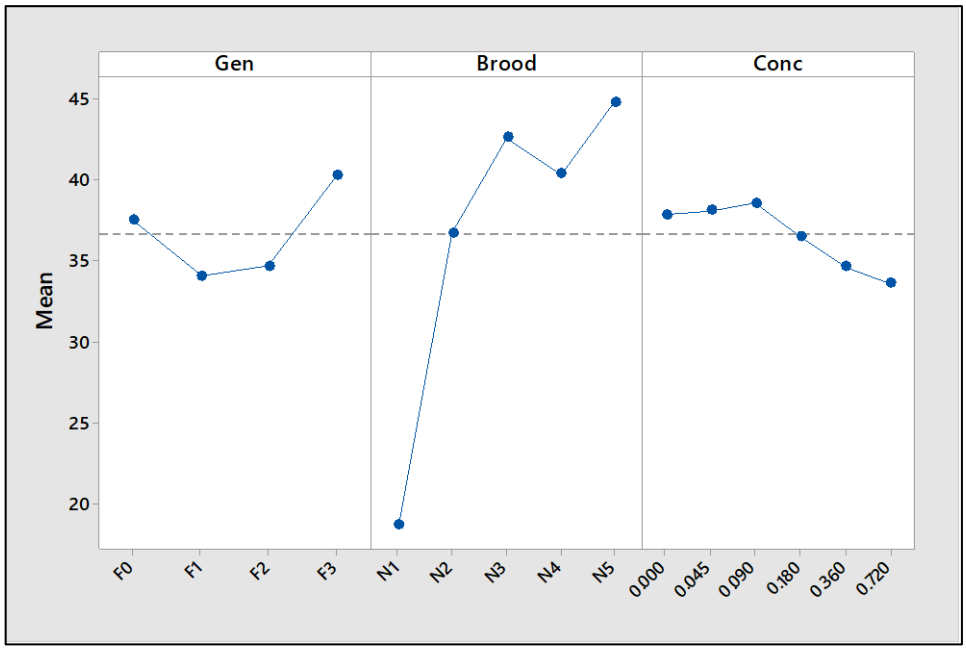

Figure 26: Main effects plots for the different generations, broods and test groups in NaClO-related reproductive success tests.

However, with all that has been said, it's important to consider that all of these tests and within all of their variables, there are deviations. The importance of this comes from the fact that tests with less deviations will be more significant than their counterparts and, therefore, should be preferentially considered. Figures 27 and 28 show the number of neonates deviations for all of the 4 tests throughout their broods, using a boxplot graphic and an interval plot with a 95% confidence interval for the mean, respectively. Despite being two different graphical representations, the overall conclusions match with one another: in all generations, as the broods progress, their mean tends to increase but also their deviations; the F0 generation is the one that presents smaller deviations when compared to all others, N3, N4 and N5 broods in F1 generation have the highest deviations in that test (especially N3), the F2 generation stands out by being the one with more deviations and the N5 brood in F3 not only was the highest deviation in that test but was also the higher deviations in all tests. The tendencies here analysed match with the descriptions previously performed but, this time, including deviations, a more accurate idea of the oscillation of the number of neonates is obtained. Also, an important factor to take into consideration with these deviations is the fact that the death of daphnids during these tests (precisely, F2 and F3) negatively influences their respective mean which, ultimately, translates to bigger deviations in that brood and concentration.

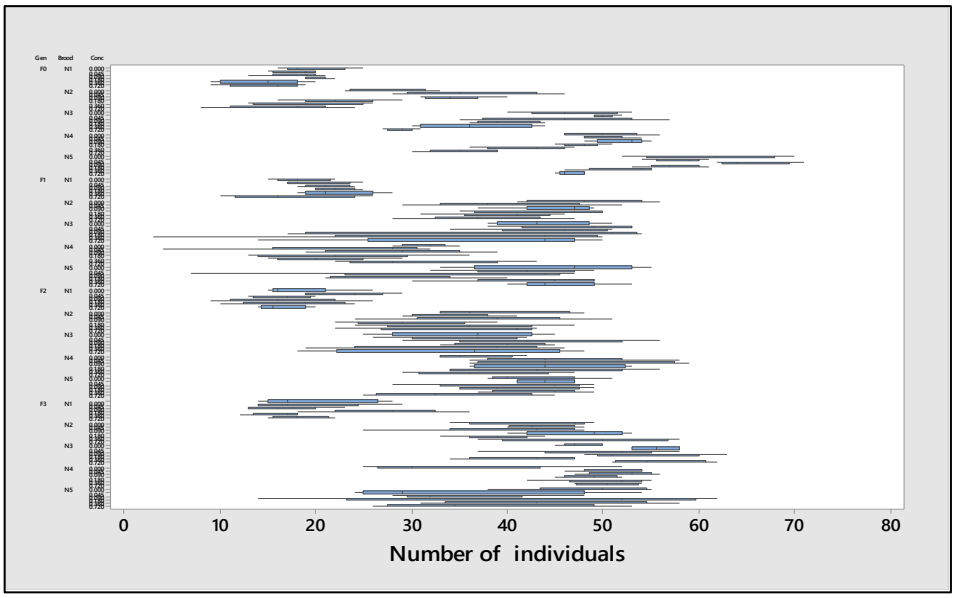

Figure 27: Boxplot of the total number of neonates for every generation, brood and concentration in the NaClO tests.

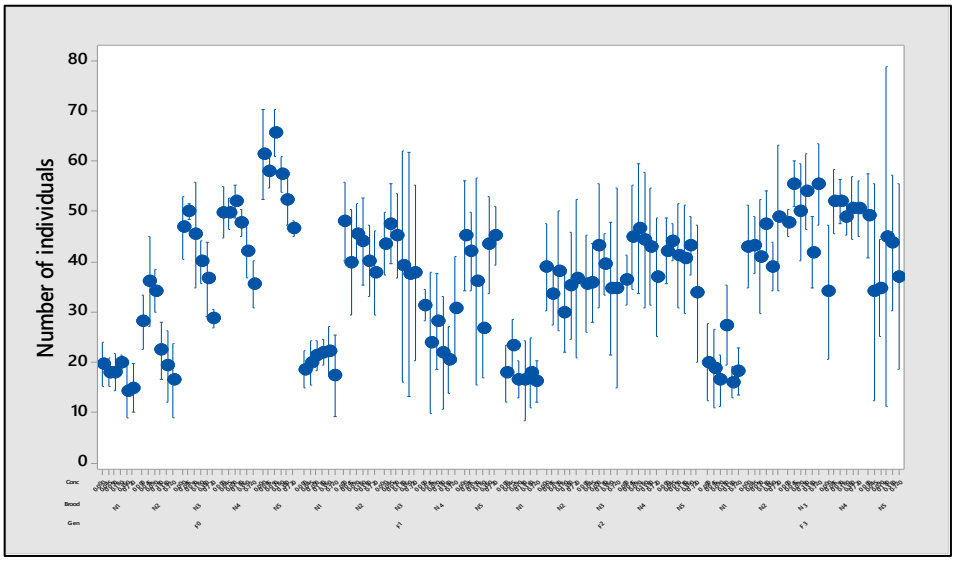

Figure 28: Interval plot of the total number of neonates with a 95% confidence interval for the mean for every generation, brood and concentration in the NaClO tests.

5.1.4.2 Body length

For the specific case of the body length endpoint in the chronic test, Figure 29 easily shows that daphnids grew steady throughout all the different broods, with the lowest concentrations having bigger daphnids at the end of the test while higher concentrations registered relatively smaller individuals.

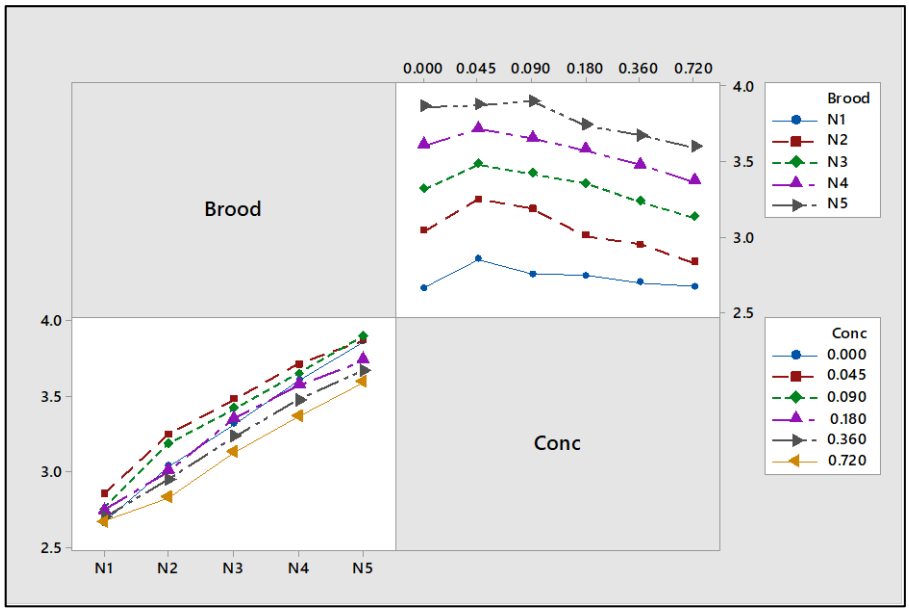

Figure 29: Summary of all the data regarding the body length daphnids exposed to NaClO. Conc: concentration.

Figure 30 also displays the same information described above, however, here it's possible to see that N1 and N2 were below the mean while N3, N4 and N5 were above it. As for the concentrations, the control and 0.18 were roughly similar to the mean, while 0.045 and 0.09 were above it and 0.36 and 0.72 were under it.

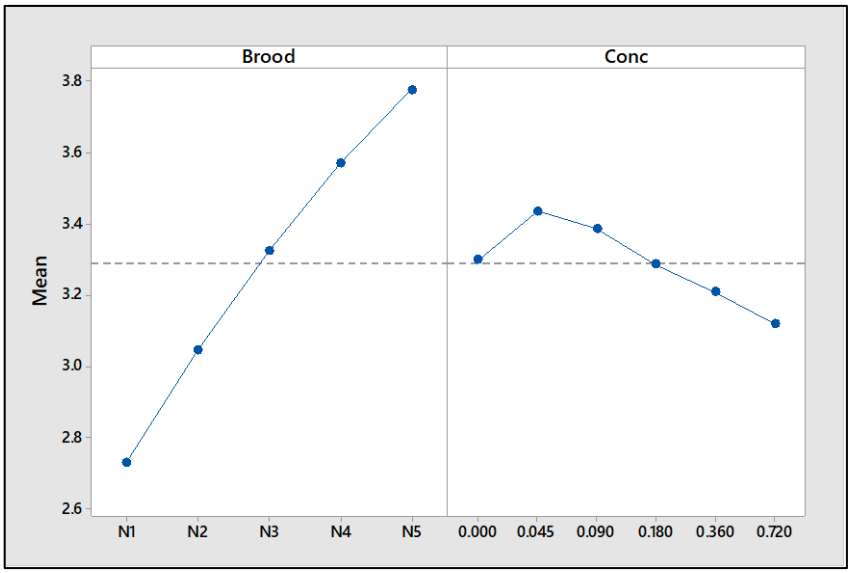

Figure 30: Main effects plots for the different broods and test groups in NaClO-related body length tests.

As for the respective deviations for this endpoint means in this test, Figures 31 and 32 show that N1 had the overall highest deviations, N2 had its highest deviations in the group test 0.72, N3 presented a higher deviation in the control group, N4 was similar to N2 and N5 was similar to N1.

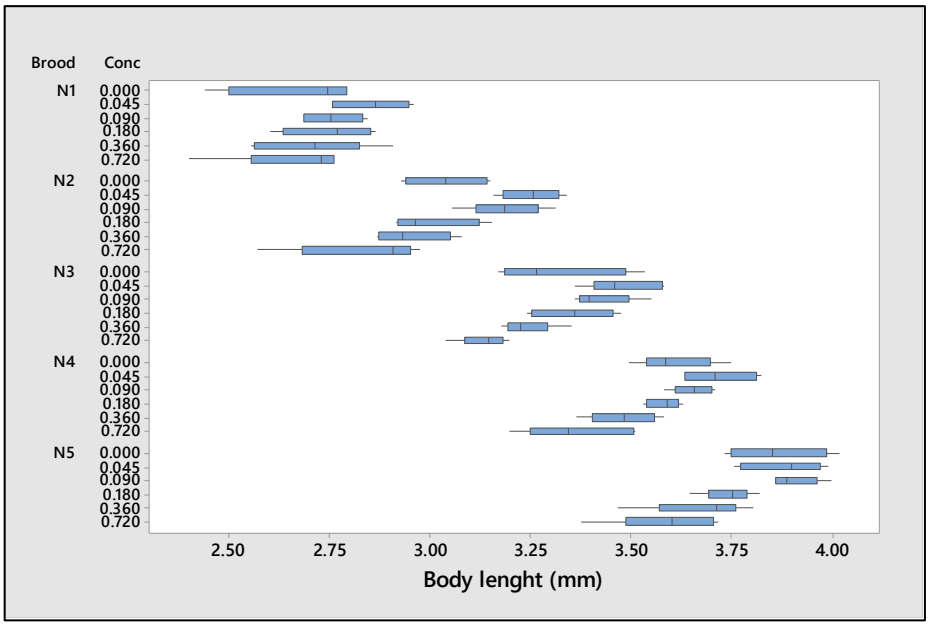

Figure 31: Boxplot of the body length sizes recorded in the chronic NaClO test in every brood and concentration.

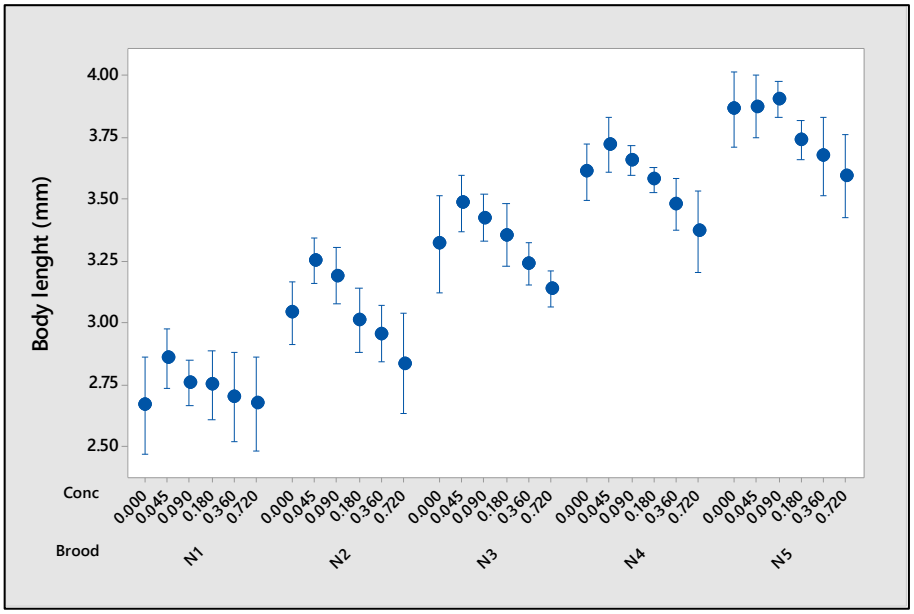

Figure 32: Interval plot of the body length size with a 95% confidence interval for the mean for every brood and concentration in the NaClO chronic test.

In order to understand if NaClO is in fact capable of inducing effects on daphnids generations that were not exposed to this contaminant, a two-way ANOVA was performed. The dependent variable is the results obtained for the different broods in all test generations. Before proceeding, it's important to say that this statistical analysis was not performed for the N1 brood because this brood is not used for acute and chronic toxicity tests therefore it doesn't make sense to employ comparisons for multigenerational and transgenerational tests. In the N2 brood, almost all comparisons were statistically significant (except for F3 vs F1): this means

that, for the N2 brood, both multigenerational and transgenerational effects exist (F0 vs all), as well as intra-multigenerational effects (F1 vs F2). In the case of the N3 brood, a pivotal brood in these tests since the subsequent tests were performed with this brood, transgenerational effects occurred (F0 vs F3). Furthermore, there seems to be a relation between both multigenerational tests and the transgenerational test (F3 vs F1 and F3 vs F2). Regarding the N4 brood, multigenerational effects existed in both tests (F0 vs F1 and F0 vs F2) but transgenerational did not. However, a statistically significance between both multigenerational and the transgenerational test (F1 vs F3 and F2 vs F3) did exist. Finally, in the N5 brood, both multigenerational (F0 vs F1 and F0 vs F2) and transgenerational (F0 vs F3) effects existed. Regarding the statistical analyses for the body length obtained in the chronic test, although there was not a statistically significant interaction between the different broods and concentrations, the comparisons between all broods were statistically significant, validating the obtained results for this endpoint.

Summarizing, when comparing to the chronic test, i.e. the exposed generation, N2 and N5 presented both multigenerational and transgenerational effects, N3 only presented transgenerational effects and N4 presented multigenerational effects in both tests.

5.2. Lethal and sublethal effects of IsoPs towards *D. magna*

Up to date, there are no available toxicity tests with IsoPs, which constitutes an important information gap. In order to bridge this gap, acute toxicity tests were performed with 8-iso-$PGF_{2\alpha}$. No immobilization was observed in all the acute toxicity tests performed. It is important to note that the lowest concentration used (0.02 $\mu g.L^{-1}$) is similar to the ones currently being reported in wastewaters (Figure 41). However, not even a 3-fold increase (32 $\mu g.L^{-1}$) was sufficient to induce immobilization. The reason why no more acute toxicity tests were performed is due to the fact that the highest concentration is already an extreme "worst-case scenario" and everything higher than that can be considered to be unrealistic. Therefore, just because no immobilization was recorded it does not mean that animals are not affected (further endpoints should be tested in the future). Furthermore, at those concentrations it is possible that changes occur when daphnids are chronically exposed.

5.2.1 Chronic test

For the chronic test, the concentrations used were the same ones used for the first acute toxicity test, which included a "real concentration", i.e. similar to the ones being reported in wastewater – 0.02 $\mu g.L^{-1}$, and several others which anticipate a possible raise in IsoPs levels in the future. The total number of neonates of the F0 test are depicted in Figure 21. When comparing to the control group, concentrations 0.02, 0.2 and 0.4 $\mu g.L^{-1}$ showed a gradual increase while 0.8 and 1.6 $\mu g.L^{-1}$ showed a decrease. This seems to indicate that, as with NaClO, small but continuous exposure to IsoPs can in fact benefit the reproductive success to a certain

point. Regarding the tendency throughout their respective broods (Figure 20), it is possible to see that the number of neonates raised from N1 to N2 and from N2 to N3 and, from N3 onwards, the number of neonates was roughly constant for each concentration. When comparing the number of neonates to the control group, we can observe that in N1, almost all concentrations gave lesser neonates (except 0.4 $\mu g.L^{-1}$), N2 and N3 all concentration groups gave more neonates and in N4 and N5, 0.8 and 1.6 $\mu g.L^{-1}$ never gave rise to more neonates than the control, 0.02 $\mu g.L^{-1}$ had slightly more neonates, 0.2 $\mu g.L^{-1}$ fewer in N4 and more in N5 and 0.4 $\mu g.L^{-1}$ always registered more neonates.

Regarding the body length (Figure 22), almost no differences occurred for the different concentrations. Nevertheless, in N1 and especially in N2 brood, the control group registered an increased daphnid length than all other concentrations. For this, we can assume that IsoPs do not interact with the growing mechanisms of daphnids and little correlate with the previous reproduction results as, throughout all the test, daphnids got bigger as the brood progressed as it was expected.

5.2.2 Multigenerational test

When it comes to the F1 multigenerational test (Figure 24), the total number of neonates for each group was always inferior to the F0-control group, meaning this one was the group that registered the higher number of neonates. Besides this, 0.02 and 0.2 $\mu g.L^{-1}$ presented lower and the lowest number, respectively, and 0,4, 0.8 and 1.6 $\mu g.L^{-1}$ registered similar numbers. The evolution of the number of neonates throughout the different broods (Graphic 14) was quite similar to the F0 test: it raised from N1 to N3, N4 was about the same as N3 and, the only different aspect, a drop in the total number of neonates in the N5 brood. For this, it also contributed the death of one replicate of the F0-0.2 test group before being able to give its respective N5 brood.

5.2.3 Overall tendencies

5.2.3.1 Reproductive success

As with NaClO, although with only two tests (generations F0 and F1), it is also important to analyse the patterns and tendencies of both tests with comparisons between their respective generations, broods and test groups. Figure 33 represents all the recorded data relative to the reproductive success in both F0 and F1 tests for its different broods and concentrations. As it has been described above, from these figures we can see that: F0 quickly increases untill it reaches the N3 brood and, for the next broods, stays steady, while F1 increases till the N4 brood and, in N5, the number of neonates goes down; F0 has a steady increasing in its number of neonates till it reaches 0.4 and then decreases while F1 had a regular number of neonates throughout all of the concentrations, with the control one registering its highest value.

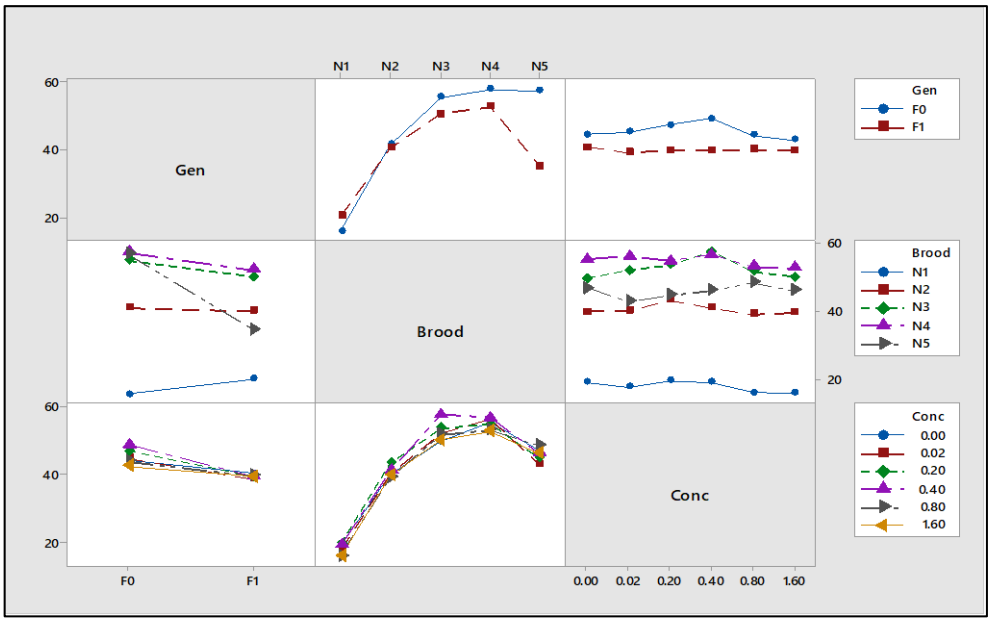

Figure 33: Summary of all the data regarding the reproductive success of daphnids exposed to IsoPs. (Gen: generation, Conc: concentrations).

In Figure 34, the main plots for generations F0 and F1, the broods and group tests are all represented. Looking to the two generations, F0 is above the mean while F1 is under the mean, which makes F0 with a higher mean of neonates than F1. For the different broods, N1 has the lowest mean of all and, along with N2, are the only ones under the mean, while N3 and N4 have high means but this decreases when it comes to the N5 brood. For the different test groups, the control stays in line with the mean but then decreases for group tests with 0.02, which goes under the mean. However, it raises for the test groups 0.2 and 0.4 (highest mean) and once again decreases for 0.8 and 1.6 (lowest mean).

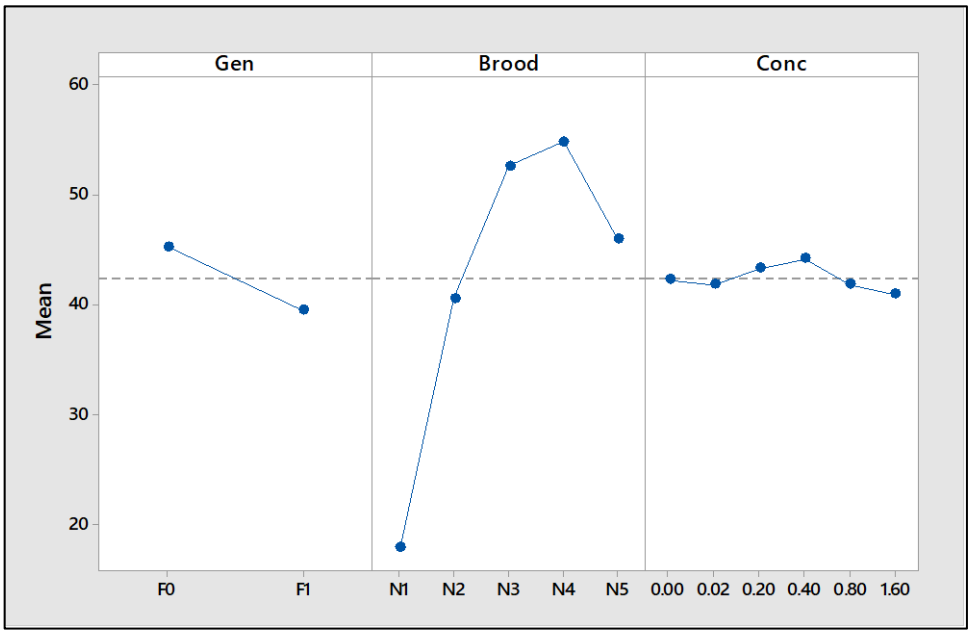

Figure 34: Main effects plots for the different generations, broods and concentrations in IsoPs-related tests.

Associated with these tests means are their respective deviations, where smaller deviations are desired since they will affect the statistical significance. Figures 35 and 36, also allow the observe the same tendency: N1 broods exhibited the lowest deviations and, as the broods progress, deviations tend to increase, with this being particularly true for the N5 brood in the F1 multigenerational test (probably due to the fact that one replicate died before it gave its neonates, as well as the high difference between concentrations that didn't happen in the other broods). Also, the tendency matches with the graphic representations previously showed.

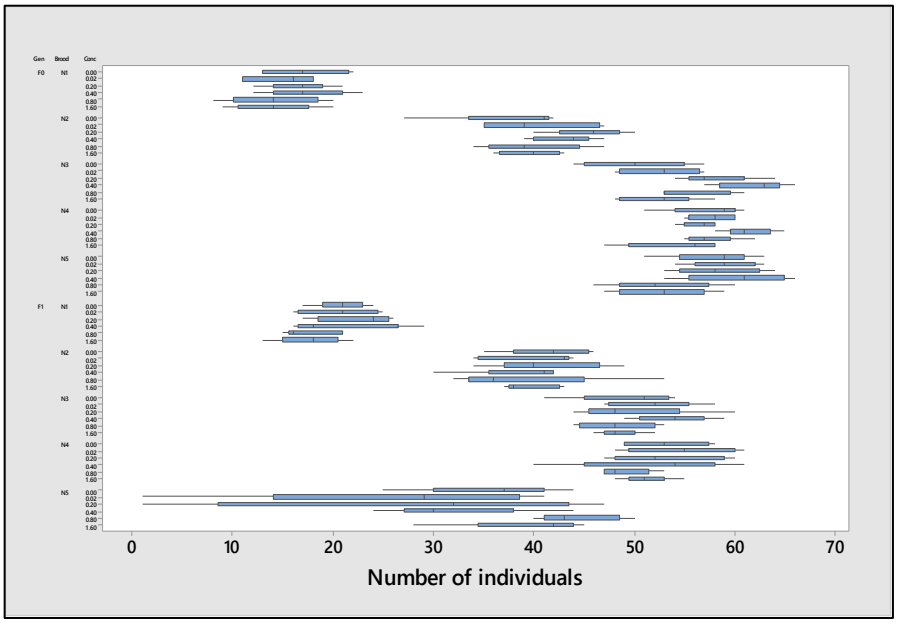

Figure 35: Boxplot of the total number of neonates for every generation, brood and concentration in the IsoPs tests.

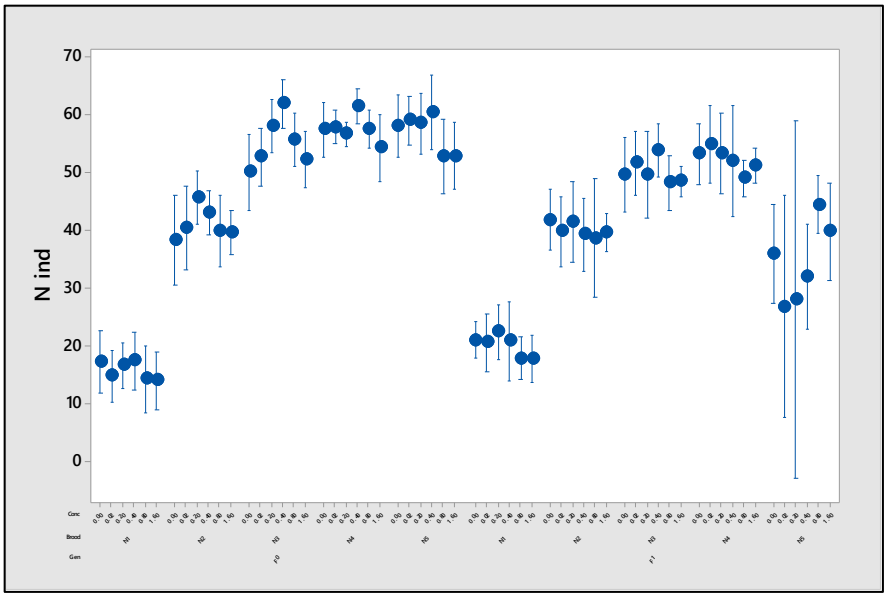

Figure 36: Interval plot of the total number of neonates with a 95% confidence interval for the mean for every generation, brood and concentration in the IsoPs tests.

5.2.3.2 Body length

When it comes to the body lengths obtained for the chronic with IsoPs, as described above, little differences were observed for all concentrations, considering the same brood. Figure 37 illustrates this, as well as the almost constant body length in all broods.

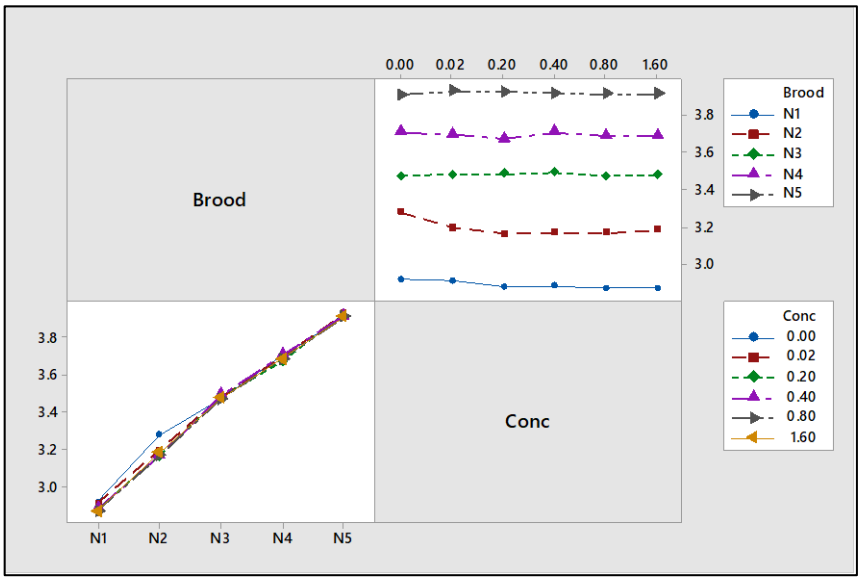

Figure 37: Summary of all the data regarding the body length daphnids exposed to IsoPs. Conc: concentrations).

Figure 38 allows to better analyse how did the means obtained for every brood and concentration group varied, when comparing to the overall mean of this endpoint.

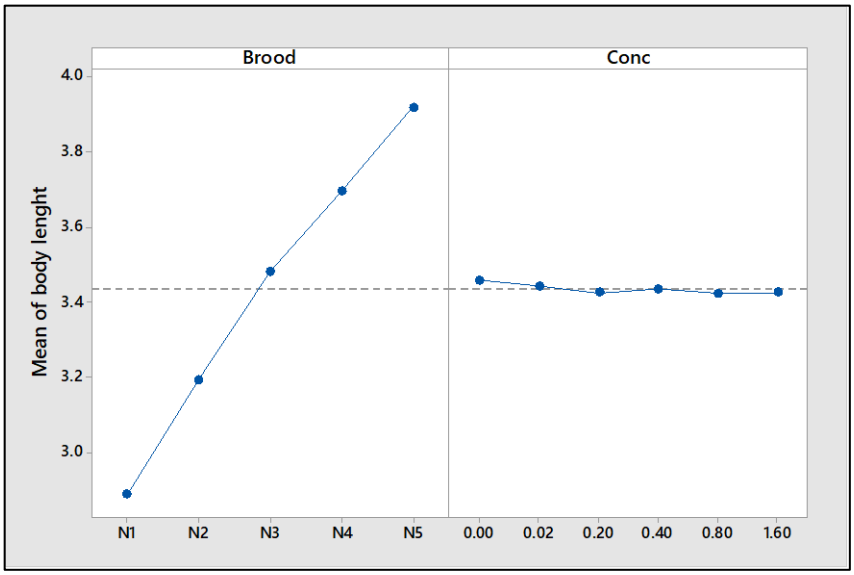

Figure 38: Main effects plots for the different broods and test groups in IsoPs-related body length tests.

Once again, as performed above, the deviations consideration are important to understand how did this test behaved statically. With both Figures 39 and 40, it is possible to observe that, due to the fact the results obtained did not practically changed between broods, only a few

punctual relatively higher deviations were registered (control group in N1, N2 and N3) and 0.4 in N2.

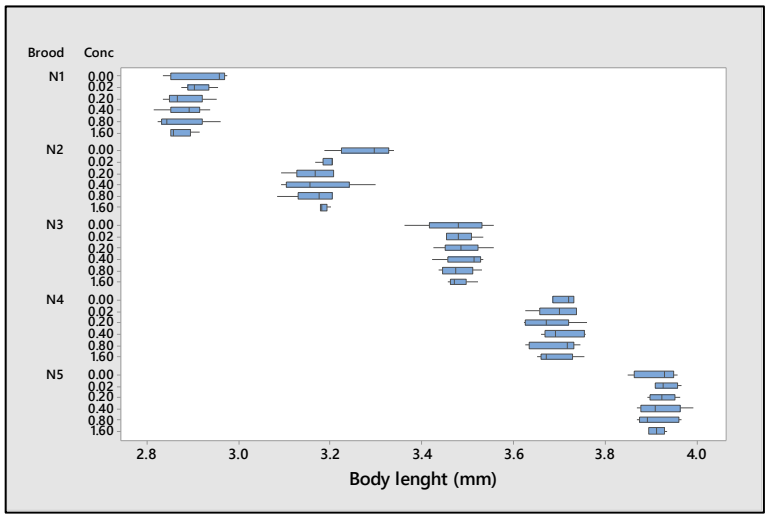

Figure 39: Boxplot of the body length recorded in the chronic IsoPs test in every brood and concentration.

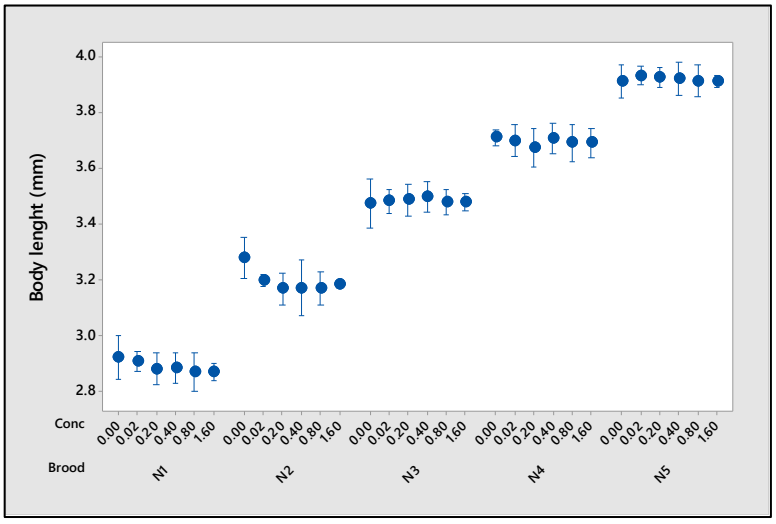

Figure 40: Interval plot of the body length with a 95% confidence interval for the mean for every brood and concentration in the IsoPs chronic test.

In order to better understand if IsoPs are also compounds capable of inducing effects in daphnid generations that weren't chronically exposed, a two-way ANOVA with the dependent variable being the number of neonates for the different broods in both F0 and F1 was performed. As with the NaClO, the N1 brood was not considered. Starting with N2, the comparison between F0 and F1 couldn't be performed since there wasn't a statistically significant interaction between both sources of variation (N2 neonates in both tests and N2 neonates in all concentrations). However, for broods N3, N4 and N5, the comparison between both F0 and F1 were statistically significant, therefore proving that IsoPs can induce multigenerational effects. When it comes to the body length statistical significance, as with the body lengths measured in the NaClO chronic test, a statistically significant interaction between the different broods and

concentrations did not exist but comparisons between all broods proved to be statistically significant.

Overall, the results from both chronic and multigenerational tests, allow to conclude that IsoPs did in fact provoke multigenerational effects in the reproductive success of *D.magna* in the N3, N4 and N5 broods.

5.3. Isoprostanes as model substances in circular toxicity

5.3.1. Isoprostanes as biomarkers in wastewater-based epidemiology

Because IsoPs are excreted from the body through urine, we can look at IsoPs as an endogenous urinary biomarker of human health (Ryu et al., 2015). Thus, given that in modern society urine is collected into the wastewater system and IsoPs allow their use as a screening method for the indirect assessment of the health of a given population, it is plausible to assume that wastewater can be used to provide a global picture of the overall oxidative stress of population.

This concept known as wastewater-based epidemiology (WBE) or "sewage epidemiology" is based on the principle that wastewater can be regarded as a large composite urine sample from a given population (Santos et al., 2015). WBE was initially proposed to evaluate the use of substances of abuse, such as illicit drugs (Zuccato et al., 2005), and it was later extended to other such substances of abuse (alcohol and tobacco), environmental contaminants and pharmaceuticals. The usefulness of WBE is by now well established, leading to its recommendation by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2016). Besides being used to estimate the use of illicit drugs in a given population, WBE has also been used to access population exposure to environmental contaminants (Been et al., 2017; Gonzalez-Marino et al., 2017; Rousis et al., 2017) and as a valuable tool to access the general consumption of pharmaceuticals and thus to indirectly study the health status of a population (Eggimann et al., 2017; Gracia-Lor et al., 2017; Rico et al., 2017; Thomas et al., 2017). The same principle can be applied to evaluate the presence of human endogenous biomarkers of disease (Daughton, 2012, 2018; Gracia-Lor et al., 2017; Ryu et al., 2016). By measuring endogenous biomarkers that provide an indication of the disease instead of measuring the pharmaceuticals used to treat those diseases it is possible to use WBE as a warning system. Furthermore, the data obtained from endogenous biomarkers can also be used to reveal health disparities between different populations as suggested by Gracia-Lor et al. (2017). Therefore, IsoPs, particularly 15-F_2-IsoPs can also be used as WBE biomarkers: they are time-integrative regarding systemic oxidative stress, are excreted in urine and are stable (Daughton, 2012). However, measurement of IsoPs levels in sewage epidemiology has not received much attention judging by the small amount of studies published (n= 4). Figure 41 summarizes the distribution of 8-iso-$PGF_{2\alpha}$ levels reported in Europe and North America, the only locations studied so far.

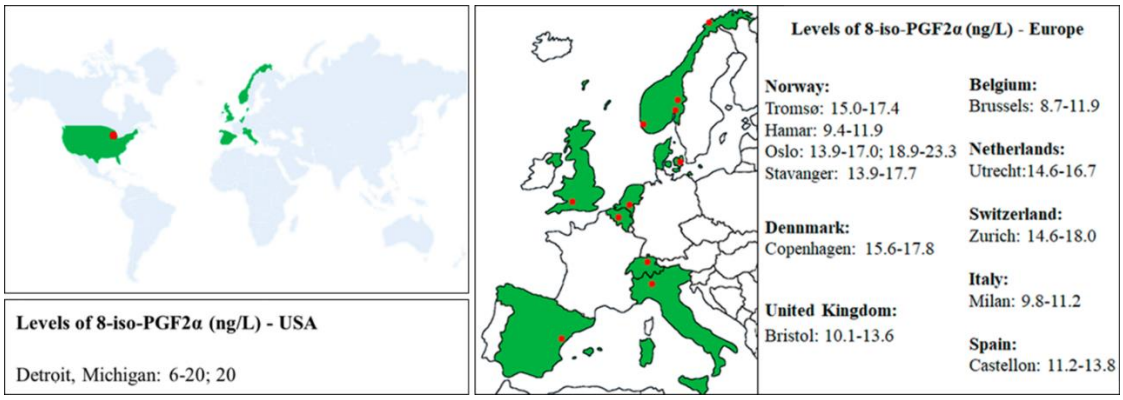

Figure 41: Geographical distribution of the studies in which environmental levels of 8-iso-$PGF_{2\alpha}$ were studied, with the indication of the detected concentrations. Data extracted from Ryu et al. (2016), Santos et al. (2015; 2016).

The first study that addressed IsoPs levels in wastewater was performed in Detroit, Michigan (USA) by Santos et al. (2015). The authors collected wastewater samples once from three different points in Detroit, between May and June of 2014 and detected 8-IsoP (a synonym to 15-F_2-IsoPs) at levels between 6-20 ng/L. Taking into account the flow rate and population, they concluded that the levels of IsoPs were community-dependent and remained constant over a period of two months. The same team (Santos et al., 2016) also quantified the levels of IsoPs in wastewater from Michigan over a period of one year between April of 2014 and May of 2015 using three different collection points, and reported IsoPs concentrations up to 20 $ng.L^{-1}$.

The subsequent studies were all performed in Europe with levels of IsoPs being described for 8 countries with a total of 11 cities. Ryu et al. (2015) reported the first concentrations while validating a new method to quantify IsoPs in wastewater by liquid chromatography–high resolution mass spectrometry coupled to an immunoaffinity clean-up. Integrative wastewater samples were collected in Oslo (Norway) in March of 2015 for eight consecutive days and the quantified 8-iso-$PGF_{2\alpha}$ levels varied between 18.9 and 23.3 ng/L. Taking into account that urinary IsoPs might be in the form of glucuronide conjugates, the authors treated their wastewater sample with ß-glucuronidase, in order to determine the total (free + conjugated) concentration of 8-iso-$PGF_{2\alpha}$. Their results showed that, when treated with this enzyme, 8-iso-$PGF_{2\alpha}$ concentration increased, confirming that IsoPs as glucuronide conjugates can go undetected. Therefore, in order to achieve maximum potential for the use of F_2-IsoPs as urinary biomarkers, it is necessary to take into account that, when excreted, a significant proportion of F_2-IsoPs will be in the form of glucuronide conjugates. This happens due to the fact that F_2-IsoPs are biologically active compounds, and in order to avoid potential adverse effects, detoxification mechanisms lead to the formation of glucuronide conjugates that will limit their activity (Yan et al., 2010). Furthermore, 15-F_2-IsoPs metabolism by the liver gives rise to several of these metabolites, of which 2,3-dinor-15-F_{2t}-IsoP and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP are those with a major presence in urine (Milne et al., 2011). Such results highlighted the complexity in the evaluation of the levels of this biomarker in real samples and strengthen the

conclusions by van't Erve et al. (2016) that total 8-iso-$PGF_{2\alpha}$ is a better indicator of oxidative stress.

The most comprehensive study up to date was also performed by Ryu and co-workers (Ryu et al., 2016). They studied the levels of IsoPs in wastewater from different European cities. The work aimed to understand if there was a correlation between the levels of IsoPs and biomarkers of alcohol (ethyl sulphate) and tobacco (trans-3′-hydroxycotinine) consumption. Previous studies demonstrated that tobacco and alcohol consumption were responsible for an increase in the relative F_2-IsoPs levels in relation to controls (Daughton, 2012). Sampling was performed over 4 or 7 consecutive days between 2014 and 2015. The results depicted concentrations of 8-iso-$PGF_{2\alpha}$ between 8.7-11.9 ng/L in Brussels (Belgium); 15.6-17.8 ng/L in Copenhagen (Denmark); 14.6-16.7 ng/L in Utrecht (The Netherlands); 11.2-13.8 ng/L in Castellon (Spain); 14.6-18.0 ng/L in Zurich (Switzerland); 10.1-13.6 ng/L in Bristol (UK); 9.8-11.2 ng/L in Milan (Italy); 9.4-11.9 ng/L in Hamar (Norway); 13.9-17.7 in Stavanger (Norway); 15.0-17.4 ng/L in Tromsø (Norway) and 13.9-17.0 ng/L in Oslo (Norway). The obtained results demonstrated that there was a strong correlation between oxidative stress and tobacco consumption from composite wastewater samples of eleven different cities. Thus, the authors suggested that the analysis of F_2-IsoPs in untreated sewage is an advantageous tool in both diagnosis and prognosis for clinical research (Ryu et al., 2016).

5.3.2. Potential toxicity of isoprostanes towards aquatic species

The same characteristics that make IsoPs a suitable WBE biomarker, i.e., their stability in wastewater (Ryu et al, 2015), ubiquity (Ryu et al, 2016) and biological activity (Daughton, 2012), may also be regarded as of possible concern towards the aquatic environment. This set of characteristics led to the proposal of IsoPs being considered as potential emerging contaminants (Gaw and Glover, 2016). IsoPs are products but also effectors of oxidative stress, and therefore as proposed by Gaw and Glover (2016), there is a possibility that exposure to IsoPs from wastewaters triggers a so called "contagious toxicity" effect that may be responsible for the propagation of oxidative stress through the aquatic environment. Therefore, as pinpointed by those authors there is an urgent need to evaluate the possible ecotoxicological impact that these compounds may have on the aquatic biota of effluent receiving waters (Gaw and Glover, 2016).

Little attention has been directed to the potential toxicity of IsoPs towards aquatic species. In fact, if one proceeds to do a quick search on Scopus, PubMed and Web of Science, to this date, there is not a single article that directly addresses the effects of IsoPs as an environmental contaminant in aquatic biota itself. The possible role of IsoPs as an emerging class of contaminants gains strength if we consider that aquatic organisms possess phylogenetically conserved prostanoid receptors (sometimes even specific receptors, e.g., the thromboxane family receptors), and given that IsoPs basically are PG-like biomolecules, they will interact with these receptors (Heckmann et al., 2008; Lang et al., 2010). The effects elicited by 15-F_{2t}-

IsoPs are mainly over the cardiovascular system, due to the fact that this compound is (i) a potent vasoconstrictor, (ii) a modulator of platelet activity, (iii) an inhibitor of angiogenesis and (iv) a promotor of atherosclerosis. These biological activities are mediated by the interaction with the thromboxane receptor (TP). Furthermore, 15-F_{2t}-IsoPs can also present biological activity through almost all of the receptors of PGE_2 (EP2, EP3 and EP4) and $PGF_{2\alpha}$ receptor (FP). It is also curious to note that, depending on which receptor 15-F_{2t}-IsoPs binds to, its activity can differ: through the TP receptor it acts as a vasoconstrictor while through EP receptors induces vasodilation (Milne et al., 2015). These specific receptors and their respective compounds are, however, mostly studied (as can be perceived from the above effects list) in the context of human medicine and clinical cases with humans and other mammals (mostly rodents) (Mizutani et al., 2010; Stanley et al., 2002). In an attempt to widen knowledge on the presence of these receptors in other species, Mizutani et al. (2010) performed a phylogenetic analysis in several eukaryotes with full sequenced genomes. From our perspective, it is important to highlight that among the species studied, both freshwater and marine organisms did indeed possess conserved PG receptors, namely *Xenopus laevis* and *Danio rerio* (vertebrates), *Ciona intestinalis* and *Branchiostoma floridae* (chordates), *Strongylocentrotus purpuratus* (echinodermata), *Nematostella vectensis* (cnidaria), *Monosiga brevicollis* (choanoflagellate) and *Trichoplax adhaerens* (placozoa).

Other aquatic organisms like the microalgae *Skeletonema marinoi* (Di Dato et al., 2017) and invertebrates like cnidarians (*Clavularia viridis, Dendronephthya sp., Dendrophyllia sp., Gersemia fruticosa, Plexaura homomalla, Telesto riisei* and *Tubipora musica*), crustaceans (*Balanus amphitrite, Carcinus maenas, Penaeus japonicus* and *Procambarus paeninsulanus*) and molluscs (*Argopecten purpuratus, Mytilus edulis, Ligumia subrostrate, Lymnaea stagnalis, Octopus vulgaris, Patinopecten yessoensis* and *Tethys fimbria*) can either produce common PGs (found in almost all of vertebrates) or novel PGs (species specific or slightly different from common PGs) (Rowley et al., 2005). Additionally, given that the COX (1 and 2) enzymes are responsible for the synthesis of PGs, it is therefore reasonable to assume that the identification of COX genes in aquatic organisms can be an indicator that these possess prostanoid receptors, similarly to what occurs in vertebrates and some invertebrates like *Gracilaria vermiculophylla* (*Rhodophyta*), *Gammarus sp.* and *Caprella sp.* (amphipod crustaceans) and *Penaeus monodon* (shrimp) (Hansen et al., 2014). Furthermore, the presence of COX genes was identified in several other specific invertebrates like *Ciona savignyi* (primitive chordate), *Daphnia pulex, Homarus americanus* and *Petrolisthes cinctipes* (crustaceans) (Hansen et al., 2014). Chung et al. (2013) reported normal levels of F_2-IsoPs and 15-F_{2t}-IsoP metabolites in the body muscle of the fish *Oryzias latipes* and Spokas et al. (2008) quantified 15-F_{2t}-IsoP in isolated *Pimephales promelas* gill pavement cells before and after exposure to lead nitrate and iron chloride and suggested the use of IsoPs as biomarkers of lipid peroxidation in aquatic toxicology studies. Given these evidences, it is more than sustained that IsoPs are biomolecules present in the aquatic biota physiology.

The cited references attest the effective evolutionary conservation of PG receptors in different taxonomic levels of aquatic biota and, most importantly, the consistency of the hypothesis that IsoPs released into the aquatic environment can elicit effects upon these species. The range of deleterious effects towards aquatic biota goes beyond those already described regarding 15-F_{2t}-IsoPs and the cardiovascular system. It has been proved that fish and invertebrates possess several PG and TX_A receptors and therefore prostanoids can modulate the immune system in these species and can also induce changes in the homeostatic processes that regulate several systems and functions such as cardiovascular, development, ion transport and reproduction (Cha et al., 2006; Gomez-Abellan and Sepulcre, 2016; Lang et al., 2010; Loof et al., 2011). Cardiovascular homeostasis interference can easily happen given that IsoPs are inductors of cardiac and vascular effects, which, ultimately, can disturb the normal development of the cardiovascular system in young fish and invertebrates. Ion transport is very important for fish, particularly to freshwater species, which need to maintain higher levels of ions in their body relatively to the medium. In this case, IsoPs interference with prostanoid receptor can lead to the disruption of the endocrine control of ion transport, furthermore, IsoPs may alter the membrane fluidity of the branchiae, key players in the transport of ions between the environment and the fish. Both these processes of cardiovascular and ionic homeostasis represent important and sensitive endpoints in the normal health of fish and it is likely that these effects are scattered throughout other phyla, with a clear example in the gills of crustaceans, that just like in fish, are sensitive to compounds that can influence membrane fluidity (Gaw and Glover, 2016).

5.3.3. Isoprostanes as effectors of oxidative stress

Unlike its usage as biomarkers of oxidative stress, there are very few studies on IsoPs as effectors of oxidative stress, and the ones available are mostly in rodent models or cell cultures. Morrow et al. (1992), explored 8-iso-$PGF_{2\alpha}$ capacity to inhibit platelet aggregation having determined a IC_{50} of 1.6×10^{-6} - 1.8×10^{-6} M. Comporti et al. (2005) tested the same IsoP at 10^{-7} to 10^{-10} M in rat hepatic stellate cells, registering the occurrence of Ca^{2+} imbalances, and markedly increased collagen and pro-inflammatory factors synthesis leading to fibrosis. In an experiment performed by Takahashi et al. (1992), 8-iso-$PGF_{2\alpha}$ at a concentration of 3.4×10^{-8} M caused renal injury in rats due to strong preglomerular vasoconstriction. Reported IsoPs levels in wastewater, specifically 8-iso-$PGF_{2\alpha}$, range between 2.5×10^{-11} – 6.6×10^{-11} M (Ryu et al., 2016). A direct comparison of these values to the effect concentrations reported by the three rat studies can, evidently be misleading. Despite the evolutionary conservation of PG receptors, the physiology of mammals diverged in multiple ways from that of fish and even further from aquatic invertebrates. However, studies reporting toxicity extrapolations between taxa, indicate for chemicals whose mode of action is receptor mediated, that both fish and invertebrates are far more susceptible than mammals (Raimondo et al., 2007). When comparing invertebrates, fish and rat chronic exposure to five pesticides, Pflüger (1995) reported between 2-6 (invertebrates), and 1-6 (fish) orders of magnitude higher sensitivity. Despite reported

current environmental concentrations of IsoPs, being orders of magnitude away from causing inhibition of platelet aggregation, fibrosis or renal injury in rat, given the much higher sensitivity of aquatic species, it is thus likely that those levels are high enough to elicit chronic responses in the aquatic biota. Furthermore, lessons learned from endocrine disruptors tell us that aquatic organisms show effects especially when continually exposed to even very low concentrations of these compounds. This happens because receptor-mediated action occurs at extremely low concentrations of chemicals — sometimes below the limits of detection for current analytical techniques — yet triggering very pronounced biological effects in a susceptible organism (Pickering and Sumpter, 2003; WHO, 2013). And just like endocrine disruptors, IsoPs also act trough receptors. As above indicated, the evaluation of the impacts of IsoPs as bioactive agents are entirely based on laboratory experimental studies with mammals. The knowledge of the ecological effects and risk assessment of IsoPs are, indeed, an urgent necessity. However, due to the low environmental concentrations of IsoPs, their chemical stability and high propensity for sub-lethal impacts, only large scale assessments are capable of unveiling the true implications of IsoPs exposure in aquatic biota. Due to their capability for a varied set of sub-lethal effects at multiple taxonomic levels, we recommend, in line with the demands of modern ecotoxicological risk assessment, a multi-tiered research strategy in order to develop a cohesive, broad temporal and spatial comprehension of the effects of IsoPs in the aquatic environment.

5.3.4. Circular toxicity hypothesis

We postulate that when humans are exposed to environmental or endogenous stressors, oxidative stress might occur leading to the production of oxidative stress markers, in which isoprostanes are included. These IsoPs are excreted trough urine that is discharged in the sewage system and will reach wastewater treatment plants (WWTPs). Because most WWTPs lack the technology to completely remove these contaminants they will enter the aquatic ecosystem. Once released into the aquatic environment, they will be available for uptake by aquatic species and because these species have receptors that are able to recognize them, they will be activated and thus the production of oxidative stress markers (i.e. IsoPs) will increase. This production of IsoPs will lead to added excretion into the environment which will in turn will promote higher uptake of these contaminants by the aquatic organisms, creating a circular toxicity loop.

The introduction by wastewaters of IsoPs in the aquatic environment and their potential effects upon aquatic species is thus matter of concern that needs to be addressed. However, establishing that IsoPs are reaching the aquatic environment and that species inhabiting it possess the receptors that will force them to interact with these chemicals does not, in itself, constitute proof of deleterious effects. The interpretation of available data from an effect concentration perspective is obligatory. Therefore, there is an urgent need to evaluate the possible ecotoxicological impact that IsoPs may have on the aquatic biota of effluent receiving

waters as already stated by Gaw and Glover (2016). Future studies should include the evaluation of sub-lethal effects of IsoPs into key species of the aquatic ecosystem, such as daphnids.

Finally, in the context of this thesis, *Daphnia* are included in the list of aquatic organisms potentially affected by the circular toxicity loop described earlier. Furthermore, given that *D. magna* and also *D. pulex* share an outlined possible pathway (through bioinformatic and transcriptomic evidence) that leads to the synthesis of $PGF_{2\alpha}$, the Daphnia $PGF_{2\alpha}$ receptors can probably also recognize IsoPs, leading to the scenario of circular toxicity. This is particularly important if we consider the core position that daphnids occupy in aquatic habitats.

For all that has been said up to this point, the complex but integrative relations between oxidative stress, sewage epidemiology and the use of *D. magna* as a model organism within the context of IsoPs is elucidated and depicted in Figure 42.

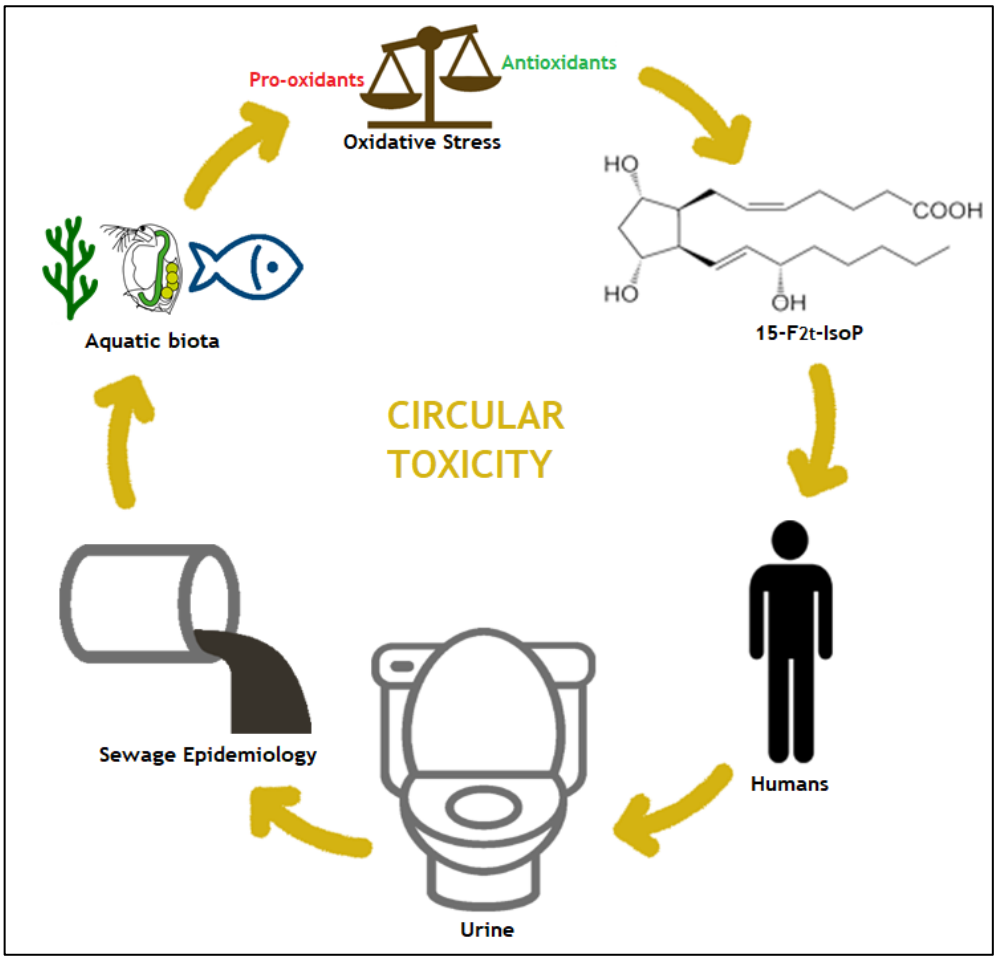

Figure 42: Circular toxicity concept representation.

Conclusions

The main objective of this thesis was to evaluate the toxicity of both NaClO and IsoPs towards the model organism *D. magna*. The results regarding NaClO from the chronic toxicity tests proved that exposure to this contaminant in high concentrations not only affected their reproductive success but also the body length in a negative way. However, for small concentrations, the inverse scenario occurred: the reproductive success and body length size of exposed daphnids were affected in a positive way, when compared to the control group. Furthermore, those effects pass into new generations as can be seen in the multigenerational and transgenerational tests performed. In order to fully understand the impact of NaClO in daphnids, future tests using different endpoints like markers of oxidative stress should be performed.

Regarding IsoPs, although no EC_{50}(48h) was determined, it did affect the reproductive success of *D. magna*. Also, considering the current reported levels of IsoPs in wastewaters and the results obtained for those concentrations, at present IsoPs will most probably not affect reproductive success. However, other tests should be done to better comprehend the real interactions and consequences of IsoPs with *D.magna* and, if relevant, with several other aquatic organisms from different trophic levels.

From a conceptual point of view, it is possible to say that in fact IsoPs are good candidates to circular toxicants, however it should be stressed that the concentrations reported in this thesis for which effects occurs are still much higher than the ones reported in the environment.

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Annex I

ASTM Hard Water

For 20L of ASTM Hard Water:

- 2400 mg $CaSO_4.2H_2O$ (stirred in distillate water before adding to the recipient);
- 4914.4 mg $MgSO_4.7H_2O$
- 3840 mg $NaHCO_3$
- 160 mg KCl

After adding these reagents to the 20L recipient, pH is adjusted between 7.6-8.0. Finally, 1mL of ASTM vitamin (previously defrosted) is added.

Annex II

MBL Medium - Woods Hole

Reference: Nichols, H. W. (1973) in *Handbook of Phycological Methods*, Ed. J. R. Stein, pp. 16-17. Camb. Univ. Press. (R. R. L. Guillard, personal communication).

Adapted for freshwater Algae

Stock solutions	Per Litre distilled water (dH_2O)	
1. CaCl2.2H2O	36.76 g	
2. MgSO4.7H2O	36.97 g	
3. NaHCO3	12.60 g	
4. K2HPO4	8.71 g	
5. NaNO3	85.01 g	
6. Na2SiO3.9H2O	28.42 g	
7. Na2EDTA	4.36 g	
8. FeCl3.6H2O	3.15 g	
9. Metal Mix CuSO4.5H2O ZnSO4.7H2O CoCl2.6H2O MnCl2.4H2O Na2MoO4.2H2O	 0.01 g 0.022 g 0.01 g 0.18 g 0.006 g	Add each constituent separately to ~750mL of dH_2O, fully dissolving between aditions. Finally make up to 1L with dH_2O.
10. Vitamin stock Cyanocobalamin (Vitamin B12) Thiamine HCl (Vitamin B1) Biotin	 0.0005 g / L dH2O 0.10 g / L dH2O 0.0005 g / L dH2O	
11. Tris stock	250.0 g / L dH2O	

Store all stock solutions in the refrigerator.

To Prepare MBL Medium

Add 1mL of each stock solution (**1 - 11**) to 1 litre distilled water. (For species which cannot use nitrate substitute 1mL of NH_4Cl made up to 5.4 g /L H2O).

Adjust pH to 7.2 with HCl.

Autoclave at 121°C (15PSI for 15 mins).

Annex III

Preparation of the seaweed extract

9mL of concentrated seaweed extract was diluted in 1L of Milli-Q water.

After that, the absorbance of this stock solution was measured at 400nm at one tenth dilution with a visible spectrophotometer – the value must be between 0.610 and 0.630. The diluted seaweed extract can be kept in the fridge protected from light.

The diluted seaweed extract is then filtered with a cellulose acetate membrane filter 0.2µm pore size. The filtered seaweed extract is then labelled and kept in the dark at 4°C.