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**Histopathological assessment of UV-
filters (TiO₂-NPs and BP-3) in
Scophthalmus maximus liver at
environmentally-relevant
concentrations**

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Histopathological assessment of UV-filters (TiO₂-NPs and BP-3) in *Scophthalmus maximus* liver at environmentally-relevant concentrations

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*“Man flows from Earth
Earth flows from Heaven
Heaven flows from Tao
Tao flows spontaneously by itself”
Tao Te Ching, Lao Tse*

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Abstract

The worldwide usage of sunscreens, formulated with organic and inorganic compounds has been increasing year by year, that was also associated with the increase of coastal and maritime tourism and the awareness of the dangers arising from exposure to solar radiation. This increase of sunscreens usage by humans raise the concern about the potential increases of the concentrations of these compounds in aquatic systems. Several studies have revealed the toxicity in aquatic organisms associated with exposure of UV-filter compounds, such as titanium dioxide nanoparticles (TiO₂-NPs) and benzophenone-3 (BP-3), however most of them do not consider environmentally-relevant concentrations of these compounds. In the present study, *Scophthalmus maximus* (turbot fish) were exposed through intraperitoneal injection to environmentally-relevant concentrations of both UV-filters, TiO₂-NPs, BP-3 and their mixture. The toxicity assessment of these compounds was performed after 3 and 7 days using histopathological analyses of turbot liver and semi-quantitative histopathological indices.

Overall, the histopathological observations of fish liver suggested a slight increase in the immune/inflammatory response after exposure to UV-filter compounds, as well as a slight intensification of the progressive alteration fat vacuolization of hepatocytes. Despite these observed alterations, no significant differences between treatments were observed and no link was found between alterations and exposure time. Through microscopic observations made using Nuclear Red (NR) staining, no nanoparticle clusters were found in the liver, suggesting that there was no accumulation of nanoparticles during the exposure time.

Summing up, this work suggested that environmentally-relevant concentrations of this compounds leads to slight alterations in turbot hepatic tissue. Nevertheless, further research about the potential effects of UV-filters compound mixtures are still needed for better understanding of possible interaction effects.

Keywords: TiO₂-NPs, BP-3, Histopathology, Inflammatory/immune response, Fat vacuolation of hepatocytes, Intraperitoneal injection

Resumo

O aumento a nível mundial da utilização de protetores solares, contendo compostos orgânicos e inorgânicos na sua formulação, está a aumentar de ano para ano. Este aumento está também relacionado com o aumento do turismo costeiro e marítimo e da consciencialização dos perigos que advêm da exposição à radiação solar. Associado a este aumento da utilização de protetores solares está a preocupação sobre o potencial aumento das concentrações destes compostos nos sistemas aquáticos. Vários estudos revelaram a toxicidade em organismos aquáticos associada à exposição a compostos de filtros-UV, como por exemplo as nanopartículas de dióxido de titânio (TiO₂-NPs) e o benzophenone-3 (BP-3), no entanto a maioria desses estudos não considera concentrações ambientalmente-relevantes. Neste presente estudo, peixes da espécie *Scophthalmus maximus* (pregado) foram expostos, através de injeção intraperitoneal, a concentrações ambientalmente-relevantes de ambos os filtros-UV, TiO₂-NPs, BP-3 e a sua mistura. A avaliação da toxicidade destes compostos foi realizada, após 3 e 7 dias de exposição, através de análises histopatológicas do fígado do pregado e através de índices histopatológicos semi-quantitativos.

De um modo geral, as observações histopatológicas do fígado sugerem um aumento ligeiro da resposta do sistema imunitário/inflamatório após a exposição aos compostos dos filtros-UV, sendo também observada uma ligeira intensificação da alteração progressiva vacuolização gordurosa de hepatócitos. Apesar das alterações observadas, não foram observadas diferenças significativas entre os tratamentos e não foi encontrada relação entre as alterações e o tempo de exposição. Através de observações microscópicas realizadas, utilizando a coloração *Nuclear Red* (NR), não foram encontrados aglomerados de nanopartículas no fígado, sugerindo que não existiu acumulação dessas mesmas nanopartículas durante o tempo de exposição.

Resumindo, este trabalho sugeriu que concentrações ambientalmente-relevantes destes compostos levam a alterações ligeiras no tecido hepático do pregado. Todavia, são necessários estudos de investigação adicionais sobre os potenciais efeitos de misturas entre os compostos dos filtros-UV para uma melhor compreensão dos efeitos dessa possível interação.

Palavras-chave: TiO₂-NPs, BP-3, Histopatologia, Resposta inflamatória/imune, Vacuolização gordurosa de hepatócitos, Injeção intraperitoneal

Index

1- Introduction	1
1.1- Problem definition	2
1.2- Organic UV-Filters focused on Benzophenone-3 and its toxicity in the aquatic environment	3
1.3- Inorganic UV-Filters focused on Titanium Dioxide-Nanoparticles and its toxicity in the aquatic environment.....	6
1.4- Histopathological analyses as a biomarker in environmental toxicology	9
2- Objectives.....	11
3- Materials and methods.....	13
3.1- Biological Model.....	13
3.2- Characterization of compounds.....	13
3.3- Experimental approach	13
3.4- Histology.....	14
3.5- Liver Histopathology	15
3.6- Statistical analysis.....	16
4- Results.....	17
4.1- Liver histopathology.....	17
4.2- Liver histopathological condition indices – semi-quantitative approach.....	21
4.3- Spearman <i>R</i> correlation analyses	24
5- Discussion.....	25
<i>Moderate inflammatory response of liver induced by sunscreen UV-filters.....</i>	<i>25</i>
<i>Slight intensification of progressive alteration (fat vacuolation of hepatocytes) due to UV-filters exposure.....</i>	<i>28</i>
<i>Assessment of TiO₂-NPs deposits in fish liver.....</i>	<i>28</i>
6. Conclusions.....	31
References.....	32

Figure Index

Figure 1.1- Efficacy range for different classes of organic sunscreen active ingredients (source: adapted from Forester, 2008).-----	4
Figure 1.2- Structure and some physico-chemical properties of benzophenone-3. (source: adapted from Mao <i>et al</i> , 2017) -----	5
Figure 1.3- Physical properties of Titanium-containing ores, namely Rutile and Anatase (source: adapted from Shaath, 2005). -----	7
Figure 1.4- Concentration (μg metal /g sunscreen) of diverse metal elements in several commercial sunscreens. (Source: adapted from Tovar-Sánchez <i>et al</i> , 2013). -----	8
Figure 3.1- Diagram of concentrations of contaminants used in bioassay and representation of treatments and exposure time.-----	14
Figure 4.1- Micrographs of liver sections of <i>S. maximus</i> (H&E stain).-----	19
Figure 4.2- (cont.) Micrographs of liver sections of <i>S. maximus</i> (H&E stain).-----	20
Figure 4.3- Comparison of the average between histopathological alterations in T_0 and all treatments. -----	21
Figure 4.4- Average of histopathological condition indice (I_h).-----	22
Figure 4.5- Average of each reaction pattern histopathological condition indices. -----	23

Table Index

Table 3.1- Condition weights from histopathological alterations observed in liver samples of <i>S. maximus</i> and respective literature reference.-----	16
Table 4.1- Spearman R correlations test results. Significant correlations ($p < 0.05$) are presented in red.-----	24

Abbreviation list

BP-3	Benzophenone-3, Oxybenzone
Fn	Focal necrosis
fv	Fat vacuolation of hepatocytes
I_h	Histopathological condition indice
I_{h1}	Histopathological condition indice for the circulatory disturbances/inflammatory response pattern
I_{h2}	Histopathological condition indice for regressive response pattern
I_{h3}	Histopathological condition indice for progressive response pattern
Li	Lymphocytic infiltration
MMCs	Melanomacrophages centres
NPs	Nanoparticles
NR	Nuclear Red
ROS	Reactive Oxygen Species
T ₀	Liver samples excised before injection of fishes
TiO ₂	Titanium Dioxide
TiO ₂ -NPs	Titanium Dioxide Nanoparticles
UV	Ultraviolet Radiation
UVA	Ultraviolet A Radiation (wavelength (nm) between 400-320)
UVB	Ultraviolet B Radiation (wavelength (nm) between 320-280)
UVC	Ultraviolet C Radiation (wavelength (nm) between 200-290)

1- Introduction

Since ancient times, human beings admire and respect the sun, not only as a source of creation and fertility but also knowing the risks that are associated with it. Thus, the sensitivity from human skin exposure to the solar radiation and the adverse problems associated with it, requires the need for its protection, either through clothing, natural paints or powders and most recently, in the late of 1920s through sunscreens (Sánchez-Quiles & Tovar-Sánchez, 2015).

With this need in mind, at the beginning of XX century, some compounds that could be used as filters of the ultraviolet radiation were found (UV-filters) and immediately the international cosmetic companies were born (e.g. L'Oreal, Delial, Piz Buin, etc.) and, consequently, making sunscreens a product available to the daily usage among the general population (Urbach, 2001; Rik Roelandts, 2008).

The Ultraviolet Radiation (UV), is part of the spectrum of electromagnetic radiation emitted by the sun and it's represented between the wavelength of 100-400 nm. According to each scientific branch, the UV is arbitrarily classified into three sections. Environmental and dermatological photobiologists usually define the wavelength regions as Ultraviolet Radiation C (200-290nm), Ultraviolet Radiation B (290-320 nm) and Ultraviolet Radiation A (320-400 nm) (Diffey, 2002). According to this division, UVC is entirely absorbed by atmospheric ozone layer and has minimal penetration to the surface of the Earth and thus has no negative effect on human health, and approximately 90% of UVB is also absorbed by ozone, whereas UVA undergoes insignificant changes when passes through the atmosphere. Therefore, the solar ultraviolet radiation of importance to human health consists of UVA and UVB (Lucas *et al.*, 2006).

The UV radiation could lead to acute and chronic adverse effects on human health; for the first case, it includes erythema, pigment darkening, vitamin D synthesis and tanning; in the second case includes, photoaging and photocarcinogenesis (Mancuso *et al.*, 2017). In general, UV radiation leads to DNA damage, however, the pathways of damage caused by UVB and UVA radiation is different; UVB radiation damage results from the formation of pyrimidine dimers and 6-4 photoproducts, whereas the predominant effect of UVA radiation is oxidative damage to DNA throughout reactive oxygen species (ROS) generated by photoactivation of endogenous photosensitizers (Marrot & Meunier, 2008). Additionally, UVB radiation can cause protein and cell membrane damage.

To prevent the effects of UV radiation on humans, UV-filters were conceived. Ideal sunscreens should have highly efficient filters against both UVB and UVA radiation and be photostable (Osterwalder *et al.*, 2009). Therefore, the most important components of the sunscreens are the UV-filters: substances with a light absorption/scattering and reflecting/diffusing ability, in the range of UVA and/or UVB, and with nearly no absorption of visible radiation linked with its commercial acceptance. There are differences in the classification of these

compounds between countries, for instance, in Europe, they are classified as cosmetic ingredients, in USA they are drugs and in Japan are classified as both (Díaz-Cruz & Barceló, 2009).

UV-filters can be classified in two types: organic UV-filters (grouped into different families: i.e. benzophenone derivatives, salicylates, cinnamates, camphor derivatives, p-aminobenzoic acid and its derivatives, etc.), and inorganic UV-filters (like metal oxides such as: titanium dioxide (TiO₂) and zinc oxide (ZnO)). These inorganic compounds used in the formulation of sunscreens are generally applied in the form of nanoparticles (TiO₂-NPs and ZnO-NPs, with size around ≤100 nm) (Osterwalder *et al.*, 2014). In addition to being used in sunscreens, UV filters can be employed in numerous cosmetics including shower gel, creams, lipsticks, shampoos, perfumes and hair sprays (Blüthgen *et al.*, 2012).

1.1- Problem definition

In a worldwide view, the awareness by the population of the risks associated with the intense exposure to sunlight and health implications of sunburns, contribute to the fastest consume and commercialization of sunscreens and other cosmetics with UV-filters. Another weight factor is the increase of coastal and maritime tourism, which is considered the fastest growing sector among the tourism industry. The trend is that the number of tourists grows every year, from 1992 to 2004 there was a worldwide increase from 463 million to 763 million and it is expected to reach 1.5 billion in 2020 (Honey & Krantz, 2007).

As a result of this growth, aquatic activities also increase and, together, the use of sunscreens was intensified. Sun care products market is an important economic sector, with € 7.0 billion forecasted only for sun protection products, estimated in 2014, and Europe accounts for 32% of the total world market, while North and South America accounts with 44% (Osterwalder *et al.*, 2014). Some of the impacts of coastal tourism in marine environment are well identified, for instance, litter pollution, degradation of ecosystems and habitat and aquifer overexploitation are very common (Tovar-Sánchez *et al.*, 2019).

Recent studies have shown that sunscreen components are able to reach the aquatic environment by two different ways: directly after being released from human skin during aquatic activities or indirectly during body washing and via wastewater treatment plants effluents (WWTP) (Giokas *et al.*, 2007; Tovar-Sánchez *et al.*, 2013; Gondikas *et al.*, 2014).

Detectable levels of UV-filters compounds were recorded in seawater, lakes, rivers, urban groundwater, swimming pools, tap water and in solid fraction samples such as sediments, sludge and sand (Li *et al.*, 2007; Sánchez-Quiles & Tovar-Sánchez, 2015). In fact, Tovar-Sánchez *et al.*, (2013) found variable concentrations of benzophenone-3 (BP-3), 4-methylbenzylidene camphor (4-MBC), TiO₂ and ZnO compounds (i.e. 53.6 - 577.5 ng/L BP-3; 51.4 - 113.4 ng/L 4-MBC; 6.9 - 37.6 µg/L Ti; 1.0 - 3.3 µg/L Zn), in nearshore waters, mainly concentrated in surface microlayer due to the lipophilic characteristic of sunscreens formulations. UV-filters can reach the aquatic environment during their entire life cycle (production, fabrication and use of products) and several studies provide evidences that these compounds can interact with aquatic organisms in several ways, such as ingestion from the sediments, adsorption to the microorganism surface and cellular internalization, leading to potential ecological risk (Baker *et al.*, 2013; Sánchez-Quiles & Tovar-Sánchez, 2015). Due to the potential toxic effects of these compounds, the European Union set the maximum concentration, allowed in the formulation of sunscreens, of 10% for BP-3 and 25% for TiO₂ (Sánchez-Quiles & Tovar-Sánchez, (2015) based on Ahmed, (2008)). As a result of these issues, the sunscreens are now classified as “emergent contaminants”, mostly because of its widespread consumption and the multitude of chemical ingredients included in their formulation and their unknown effects (Tovar-Sánchez *et al.*, 2019).

The toxic effects of UV-filters have been demonstrated in various aquatic species from phytoplankton and marine algae to invertebrate and vertebrate organisms. Growth inhibition, oxidative stress and hormonal alterations, are the most common reported effects (Baker *et al.*, 2013; Tovar-Sánchez *et al.*, 2013; Sánchez-Quiles & Tovar-Sánchez, 2015; Tovar-Sánchez *et al.*, 2018). To sum up, the worldwide use of sunscreens is increasing each year and consequently the input of organic and inorganic compounds on aquatic habitat is being intensified. Due to this, the importance of knowing the hazards and concerns around the effects of sunscreen compounds and their influence on organisms of marine fauna and flora is essential, so it's extremely important the need of more studies and experimental approaches in order to fill the gaps and find solutions.

1.2- Organic UV-Filters focused on Benzophenone-3 and its toxicity in the aquatic environment

Nowadays there's allowed by different legislations fifty organic compounds to be used as UV-filters in sunscreen composition (Sánchez-Quiles & Tovar-Sánchez, 2015). The ability of organic filters to absorb UV radiation is mainly in the active ingredients that absorb UV energy to a various extent within a specific range of wavelengths depending on their chemical structure. Absorbed energy is converted into imperceptible infrared (heat) energy. Chromophore is the molecular structure responsible for absorbing UV energy and it consists of electrons engaged into multiple bond sequences between atoms, generally conjugated double bonds (Forestier, 2008).

According to their UV absorption range, organic UV-filters are classified either as UVB or UVA filters, but cannot cover both sides of spectrum, as shown in figure 1.1.

At the atomic scale, the functionality of organic filters is briefly explained as an absorbed UV photon by the chromophore, that holds enough energy to cause electron transfer to a higher energy orbit in the molecule (Kimbrough, 1997). Simplifying, the molecule that was in a low-energy state (ground state) transforms into a higher-energy state (excited) and from this excited state and depending on the capacity of the UV-filter to process and convert the absorbed energy, different relaxation processes could happen (Forestier, 2008):

- (i) Photostable: The filter molecule may simply deactivate from its excited state to its ground state while releasing the absorbed energy to the environment in the form of heat. The filter fully recovers its ability to absorb UV photon repeatedly.
- (ii) Photounstable: Degradation or structural transformation may occur, and the filter loses its absorption capacity and protective potency quickly.
- (iii) Photoreactive: The excited molecule can interact with its surroundings, other ingredients contained in sunscreen product, ambient oxygen, or skin biomolecules (eg: proteins, lipids) and, thus, lead to the production of undesirable reactive species.

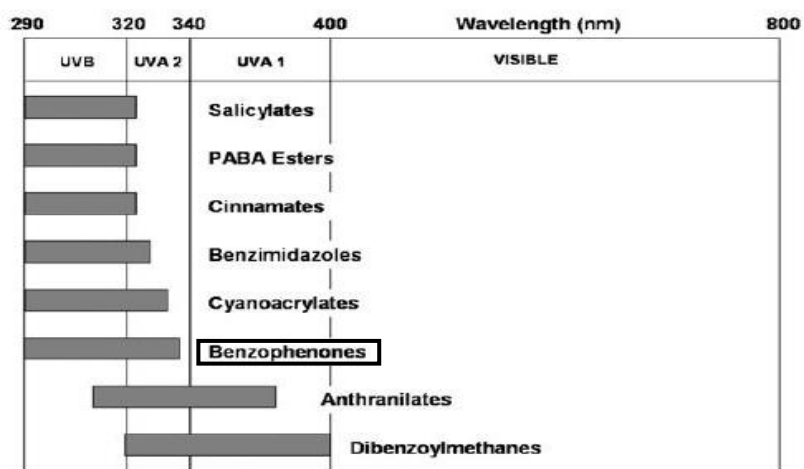


Figure 1.1- Efficacy range for different classes of organic sunscreen active ingredients listed in Food and Drug Administration monograph. Special focus on Benzophenones compounds (source: adapted from Forester, 2008).

As previously mentioned, the compounds of organic filters can be: (i) photostable, (ii) photounstable or (iii) photoreactive. They can be degraded in some different ways. By natural mechanism of photodegradation, like photolysis (which sunlight-induced photochemical reactions are the driving forces), photoisomerization, disinfection, break down by-products in WWTP, or even excreted in urine (after skin absorption they “travel” until kidneys, where the compounds are metabolized and transformed into metabolites) (Díaz-Cruz *et al.*, 2008).

Among organic compounds of sunscreen formulations, Benzophenones compounds are the only chemical class that belongs to the aromatic ketone category (Shaath, 2005). Between the Benzophenone family, the most frequently used in sunscreens formulation, is the Oxybenzone (Benzophenone-3), due to its stable molecules that allows the absorption essentially in UVB spectrum side, though with some absorption in the UVA 2 (Mancuso *et al.*, 2017). Their physicochemical properties are represented in figure 1.2.

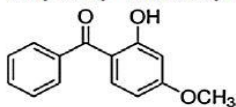
Properties	Benzophenone-3
Synonym	2-Hydroxy-4-methoxybenzophenone, Oxybenzone
Chemical structure	
Molecular formula	C ₁₄ H ₁₂ O ₃
CAS. number	131-57-7
Molecular weight (g mol ⁻¹)	228.24
Water solubility (mg L ⁻¹)	2295.40 ^a
logK _{ow}	3.79 ^a
pKa	7.56 ^b
Half-life in surface water (h)	502.00 ^a

Figure 1.2- Structure and some physico-chemical properties of benzophenone-3. (source: adapted from Mao *et al.*, 2017)

In spite of degradation, some organic UV-filters (e.g. BP-3, octocrylene (OCR), PABA) have toxic properties and it has been demonstrated that under certain conditions they can generate reactive oxygen species (ROS), like superoxide (O₂⁻), hydroxyl (OH) and hydrogen peroxide (H₂O₂). ROS can induce damage in lipids, proteins, and DNA and in some marine organisms the effects are associated with high oxidative stress levels (Sánchez-Quiles & Tovar-Sánchez, 2015).

Some studies have reported and evaluated the toxicity associated with several organic UV-filters, including the BP-3. In a recent study, Chen *et al.*, (2018) concluded that the survival and growth of false clown anemonefish (*Amphiprion ocellaris*) were not affected by the BP-3 exposure (chronic dietary exposure of 1 000 ng BP-3/g food), as well as the intra-colonial social behaviour. Another study focused on assessing the estrogenic activity and reproductive effects of BP-3 on rainbow trout (*Oncorhynchus mykiss*) and Japanese medaka (*Oryzias latipes*), reporting that the exposure to BP-3 induced vitellogenin in both species. However, the concentrations (620-749 µg BP-3/L) were significantly higher than concentrations (~20 ng BP-3/L) usually found in wastewater effluents, suggesting that oxybenzone may not be a significant risk to fish reproduction and that the contribution to estrogenic activity is limited (Coronado *et al.*, 2008). Another study assessed the oxidative stress and estrogenic effects of BP-3 in zebrafish (*Danio rerio*) and the results suggested that 1 000 µg BP-3/L induced a significant increase in vitellogenin relative expression. No effects in transcription levels of catalase, glutathione peroxidase and superoxide dismutase were observed after acute exposure to BP-3 (Rodríguez-Fuentes *et al.*, 2015). Blüthgen *et al.*, (2012) reported alteration of genes involved in steroidogenesis and

hormonal pathways after exposure to low concentrations of BP-3 (2.4 - 312 µg BP-3/L) in zebrafish (*Danio rerio*) demonstrating that the effects occurred mainly at 84 µg BP-3/L. Nevertheless, at histological level, no effects were observed.

1.3- Inorganic UV-Filters focused on Titanium Dioxide-Nanoparticles and its toxicity in the aquatic environment

As mentioned previously, there are two main pigment grade powders of metal oxides used in the sunscreen formulation, TiO₂ and ZnO, which are used combined with organic filter to improve the protection level in the longer UVA range (>340 nm) and simultaneously boost the sun protection factor (SPF) values. The inorganic UV-filters are able to scatter the UV rays and increase their probability to interfere and hit with the molecules of organic UV filters compound. Unlike organic filters that absorb UV radiation, inorganic filters work by reflecting and diffusing UV radiation (Shaath, 2005; Forestier, 2008; Lionetti & Rigano, 2017) (18).

Titanium (Ti) is the ninth most common element in the earth's crust. In nature, it exists only in combinations with other elements and at commercial field three titanium-containing ores are important: rutile, anatase and ilmenite. Therefore, commercial TiO₂ is always synthesized (Shaath, 2005). Titanium dioxide is efficient, almost photostable, absorb the UVB and UVA radiations and re-emit them mainly as visible wavelength or heat. Due to its physical-chemical characteristics, the surface is often coated with other materials, considered as "inert" substances like silica, alumina, stearic acid or silicone compounds (Shaath, 2005; Lionetti & Rigano, 2017). Titanium dioxide is especially used in "nano-sized", because nanosized dimension enhances the protection capacity, so the product could be used in a large range of materials, it's then called TiO₂-NPs (titanium dioxide nanoparticles) (Tyner *et al.*, 2011). As mentioned before, commercial TiO₂ is always synthesized and the production of nano-sized TiO₂ are typically made from the "sulphate process", that is used by the most producers, where titanium ores are reacted with sulfuric acid resulting in a titanyl sulphate intermediate (Shaath, 2005). It was estimated, that the global production of that material (TiO₂-NPs) is around 550 to 5 500 tons per year (Piccinno *et al.*, 2012). Figure 1.3 shows the physical properties of the two most important forms of TiO₂ (rutile and anatase), which have the same chemical identity, differing in their crystalline structure. Moreover, TiO₂ is very stable, insoluble in organic solvents and essentially inert in all applications (Shaath, 2005).

Parameter	Rutile	Anatase
Density (g/cm ³)	4.2	3.9
Hardness (Mohs)	6–7	5.5–6
Refractive index	2.76	2.52
Dielectric constant	114	48
Melting point (°C)	1855	Converts into rutile

Figure 1.3- Physical properties of Titanium-containing ores, namely Rutile and Anatase, with commercial importance (source: adapted from Shaath, 2005)

The ability of inorganic metal oxides to act as UV-filters is determined by two major characteristics: the property of absorption/scattering the UV radiation and its cosmetic acceptability (if they leave an undesired whitish appearance in the skin or not) (Serpone *et al.*, 2006), which are associated with those particle sizes. The smaller the particles, greater homogeneity applied over the skin, providing better coverage of the epidermis. Moreover, for particle size of about 35 nm, TiO₂-NPs absorb, scatter and reflect UV radiation in short-wavelength, while in the longer-wavelength, as visible light, they keep it invisible and the sunscreen formulation is transparent to the eyes. The optimum particle size for good transparency and elevated UVB and UVA attenuation is between 40 nm and 60 nm (Lionetti & Rigano, 2017).

Thus, it became evident for TiO₂ that (adapted from Shaath, 2005):

- UVB attenuation is predominately due to its absorption, which increases as the particle size decreases.
- UVA attenuation is predominately due to scattering, which increases as the particle size gets larger. So, particle size needs to be controlled to maximize the attenuation without causing whitening.

The concentration of TiO₂-NPs in commercial sunscreens depends from brand to brand. Tovar-Sánchez *et al.*, (2013) analysed thirteen commercial sunscreens and the concentration of several metals (µg metal /g sunscreen), including titanium (Ti) element, is depicted in figure 1.4.

Sunscreen	Al	Fe	Ti	Zn
1	nd	nd	11146 ± 401	nd
2	342 ± 27	68.7 ± 3.7	nd	nd
3	nd	nd	nd	nd
4	nd	nd	6302 ± 973	nd
5	nd	nd	nd	nd
6	1071 ± 93	nd	14855 ± 1774	nd
7	nd	nd	nd	72688 ± 101
8	nd	nd	12308 ± 1075	nd
9	nd	nd	43126 ± 2105	nd
10	862 ± 86	nd	12381 ± 1417	nd
11	nd	nd	23108 ± 891	nd
12	nd	nd	37256 ± 1456	nd
13	nd	nd	46507 ± 1788	nd

(nd) not detected

Figure 1.4- Concentration ($\mu\text{g metal/g sunscreen}$) of diverse metal elements in several commercial sunscreens. Special attention for Titanium element (Source: adapted from Tovar-Sánchez *et al.*, 2013)

The toxicity of TiO_2 -NPs varied with different species, particle sizes, and exposure conditions, like concentration and exposure time (Hou *et al.*, 2018). The mechanism of toxicity of TiO_2 -NPs is usually defined in three aspects: (1) The reactive oxygen species (ROS) produced by TiO_2 -NPs following the induction of electron-hole pairs created by photocatalytic activity (Handy *et al.*, 2008; Wang *et al.*, 2016; Hou *et al.*, 2018). (2) Cell wall damage and lipid peroxidation of the cell membrane (cytotoxicity) caused by NP-cell attachment by electrostatic force owing to the large surface area of TiO_2 -NPs (Labille *et al.*, 2010; Wang *et al.*, 2016; Hou *et al.*, 2018). (3) Cytoplasm flow out and TiO_2 -NPs attachment to intracellular organelles and biological macromolecules following damage to the cell membranes (Applerot *et al.*, 2009; Hou *et al.*, 2018).

Some studies demonstrated that the toxicological effects of TiO_2 -NPs may represent a potential risk to different marine organisms (algae, invertebrates and vertebrates). Toxicity was reported in phytoplankton, that belongs to the base of trophic food chain, where Handy *et al.*, (2008) and Wang *et al.*, (2016) assessed that TiO_2 -NPS can inhibit the growth of freshwater and marine phytoplankton through the production of high levels ROS. Vignardi *et al.*, (2014), found that TiO_2 -NPs cause genotoxicity in the erythrocytes of juvenile *Trachinotus carolinus*, leading to DNA damage but didn't cause fish mortality. The same authors also reported that TiO_2 -NPs has cytotoxic potential and that cellular uptake may occur in kidney, liver, muscles and gills from that species. Souza *et al.*, (2018) reported that TiO_2 -NPs was internally sequestered in *Centropomus parallelus* cells due to environmental contamination and pointed that the localization of TiO_2 -NPs was in cytoplasmic vesicles and in the nucleus of cells suggesting a possible route of sequestration and detoxification. On the other hand, Federici *et al.*, (2007) showed that TiO_2 -NPs caused respiratory toxicity, as well as disturbances in the metabolism of some trace elements like Zn and Cu in rainbow trout (*Oncorhynchus mykiss*).

The same authors also found that TiO₂-NPs induces oxidative stress without appreciable titanium accumulation in the internal organs in short term. TiO₂-NPs also induced histological alterations, showing condensed nuclear bodies (apoptotic bodies) and minor fatty change in liver after short time exposures (Federici *et al.*, 2007). On the contrary, Scown *et al.*, (2009) reported that high doses of intravenously injected TiO₂-NPs induced very limited (if any) overt impairment of renal function or oxidative stress in the blood in rainbow trout, despite the evidence of significant uptake and retention in this tissue, which can bioaccumulate over time. Boran *et al.*, (2016) observed that TiO₂-NPs can adsorb toxicants like Hg²⁺ and this association may alter the delivery of toxicants to organisms located in specific environmental compartments.

1.4- Histopathological analyses as a biomarker in environmental toxicology

Histopathological analyses are a very useful biomarker in environmental toxicology because it is considered a more realistic tool than biochemical approaches to assess toxicity (Handy *et al.*, 2002; Wester *et al.*, 2002; Stentiford *et al.*, 2003; Au., 2004). Histological responses can be related to real health state of individuals organism, which allows better extrapolation to population/community and consequently estimates the potential effects of environmental contamination (Au, 2004; Costa *et al.*, 2011). Qualitative histopathological approaches have provided vital information on the description of histological lesions and alterations in field-collected or tested aquatic organisms, and it gives some practical advantages like easiness of sample collection and storage, and the ability to assess many body systems and cell types from the same fish. Still with respect to aquatic environments, the fish liver has been considered one of the major targets of assessment due to its function in xenobiotic transformation, storage and, even, elimination of chemicals (Handy *et al.*, 2002; Costa *et al.*, 2009; Costa *et al.*, 2011).

Nevertheless, integrating biological data with environmental parameters and establishing cause-effect relationships between pathology and contamination patterns is difficult through only qualitative approaches, as well as assessing the significance of the differences between surveyed groups, due to absence of numerical data. Based on this issue, several authors developed histopathological indices to provide numerical data based on a semi-quantitative approach, employing multivariate statistics using lesion frequency indices and weighted indices based on lesion progression and considering that different lesions may not have the same biological impact in the organism. Thus, linking qualitative and quantitative approaches and conferring also a broader biological significance (Van Dyk *et al.*, 2007; Costa *et al.*, 2011; Martins *et al.*, 2016).

However, this biomarker isn't yet a rule and there are some difficulties associated with. One of the most important difficulties of histopathological studies in fish relates to the lack of specificity of lesions and changes towards a contaminant or class of contaminants, which greatly impairs cause-effect assessments when multiple toxicants are involved and the lack of consensus between histopathological terminology and identification features (Costa *et al.*, 2009; Costa *et al.*, 2011). Still, there are yet insufficient studies (trend to increase) with fish exposed to environmentally realistic concentrations of sunscreens compounds or only about a specific compound and even fewer concerning histopathology derived from those compounds.

2- Objectives

The main goal of the present Thesis is to assess the acute histopathological effects and alterations, induced by the exposure of environmentally-relevant concentrations (3.0 µg contaminant /g fish) of both inorganic and organic commercial sunscreens compounds in *Scophthalmus maximus* liver. For this purpose, two mostly used UV-filters were selected as models, the TiO₂-NPs, BP-3 and their mixture.

The specific objectives were:

- I. Assessing the effects of individual UV-filters;
- II. Assessing the interaction effects of both UV-filters (mixture exposure);
- III. Evaluating the time-response effect;
- IV. Identifying possible TiO₂ nanoparticles presence in fish tissue.

3- Materials and methods

3.1- Biological Model

Scophthalmus maximus (turbot) was chosen as a model organism due to its ecological and economic relevance. The turbot belongs to *Scophthalmidae* family and *Scophthalmus* genus. Naturally, it is widely distributed in European waters, from Northeast Atlantic to the Arctic Circle and in the Mediterranean Sea (Danancher & Garcia-Vazquez, N.d). Is a benthic and coastal marine species, living on sandy and muddy bottoms from shallow waters to 100 m. When juvenile, its diet is based on crustaceans and in adult stage feed mainly on other bottom-living fishes and cephalopods (FAO, 2012). These behaviour and habitat characteristics are relevant, once contaminants tend to income mainly from coastal zones, with tendency to sediment in bottom layers, increasing the contaminants contact probability with benthic species (Costa *et al.*, 2011). The commercial farming of turbot has grown steadily in the Atlantic region (Garza-Gil *et al.*, 2009; Nie *et al.*, 2019). It's a species widely produced in aquaculture throughout Europe, and the main producer countries are Denmark, France, Germany, Spain, UK and Portugal (FAO, 2012). European production was estimated around 5 000 tonnes per year (Danancher & Garcia-Vazquez, N.d).

3.2- Characterization of compounds

The compounds used in the assay were supplied by Sigma-Aldrich. Titanium Dioxide Nanoparticles (TiO₂-NPs), namely Aeroxide®P25, with declared purity ≥ 99.5% and CAS#13463-67-7 and Benzophenone-3 (BP-3, 4-methoxy-2-hydroxybenzophenone, oxybenzone) with purity = 99% and CAS# 131-57-7. The TiO₂-NPs suspensions were prepared in NaCl by sonication with an ultrasonic processor (Sonics vibra cell), during 15 minutes at 100 W, with 5:1 pulses on/off. The dispersion was performed in an ice bath. BP-3 solution was prepared in DMSO and NaCl solution.

3.3- Experimental approach

The *in vivo* bioassay was performed in the University of Aveiro with authorship from CESAM, under controlled conditions.

Fish with a total weight 19.6 ± 3.37 g, were obtained from a local aquaculture (Acuinova, Portugal) and acclimatized for 15 days. After this period, a total of 15 fish/treatment were injected as described in figure 3.1.

- Control - (injection with NaCl and 10 μ L of DMSO);
- Treatment A - (injection with 3.0 μ g of TiO₂-NPs /g of fish);
- Treatment B - (injection with 3.0 μ g BP-3 /g of fish);
- Treatment C - (injection with a mixture of 3.0 μ g TiO₂-NPs + 3.0 μ g BP-3 /g of fish).

All treatments had two exposure times, T₃= 72h and T₇= 168h. Since the injected BP-3 solution contained 10 μ L of DMSO and TiO₂-NPs was prepared in NaCl, all fish from control group were injected with NaCl and DMSO, to eliminate the possibility of these compounds interferences. Each treatment consisted of three 5 L aquariums each containing 5 fish (15 fish per condition – 1L of water/fish). In total there were 24 aquariums, all with identical water conditions: oxygen between 7.4 \pm 0.8 and 7.7 \pm 0.6 mg/L, pH between 8.09 \pm 0.17 and 8.17 \pm 0.2, NH₃ between 0 and 0.25 mg/L, temperature = 17.3 \pm 0.6 $^{\circ}$ C and salinity = 35.

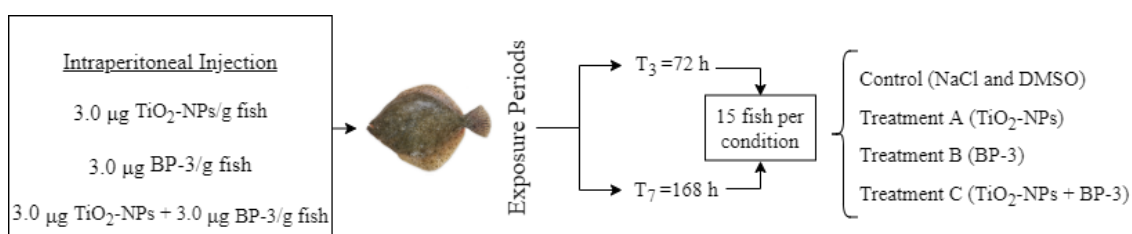


Figure 3.1- Diagram of concentrations of contaminants used in bioassay and representation of treatments and exposure time. Treatment C was a mixture of BP-3 and TiO₂-NPs.

To simulate contamination through ingestion, fish were injected intraperitoneally with 3.0 μ g of contaminant/g of fish. The injected concentrations are environmentally-relevant and in agreement with data found in the literature (Scown *et al.*, 2009; Vignardi *et al.*, 2015).

Before the injection, 8 fish were excised, and liver samples were immediately fixed (T₀ liver samples). After 72h (T₃) and 168h (T₇) of exposure, 9 fish per treatment were excised and liver samples were immediately fixed in Bouin-Hollande solution for 48 h. Afterwards, the samples were washed with distilled water and, finally, archived in ethanol 70% v/v.

3.4- Histology

According to Martins *et al.*, (2016), the samples were submitted to dehydration with 2 progressive series of ethanol 95% (15 minutes each) and 3 series of ethanol 100% (30 min each), then 3 series of intermediate infiltration with xylene (15 min each) and were quickly infiltrated in liquid paraffin. After obtaining solid paraffin blocks with samples within, liver sections of 5 μ m thickness were obtained. At least, 10 sections per slide, using the manual rotary microtome (Leica JUNG RM2035) were achieved.

Afterwards, were staining with the Haematoxylin (for 4 min) and counterstained with basophilic alcohol eosin (for 1 min) (H&E stain) for general structural analyses. A low intensity staining was made, using Nuclear Red (NR) a hydrophilic dye appropriated for counterstain metal deposits, which was used to identify nanoparticles (Fernandes *et al.*, 2017). Slides were progressively dehydrated with 2 times ethanol (95% and 100% v/v) and xylene, for 30 seconds each. After dehydration, slides cleaned with one drop of xylene and then mounted with DPX mounting medium.

3.5- Liver Histopathology

Observations were performed with a Leica DMLB microscope, equipped with a DFC480 digital camera. Image processing and analysis was achieved using the IrfanView software. Semi-quantitative histopathological condition indices were estimated for each individual liver sample. The histopathological alterations, adapted from Bernet *et al.*, (1999) and Costa *et al.*, (2009), were divided into three reacting patterns, namely, circulatory disturbances/inflammatory response, regressive and progressive changes. For each alteration was assigned a condition weight (w) that ranged between 1 (low severity) to 3 (high severity) and the degree of dissemination of the alteration within the surveyed organ (score) ranged between 0 (absent alteration) to 5 (diffuse alteration). In table 1 is represented the list of the alterations that were observed, and the ones considered for the estimation of histopathological indices (with *) and their respective biological importance (weight, w). The indices were estimated through the formula (1) proposed by (Costa *et al.*, 2013):

$$(1) \quad I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

Where I_h is the histopathological condition indice for the individual h ; w_j the weight of the j th histopathological alteration; a_{jh} the score attributed to the h th individual for the j th alteration and M_j is the maximum attributable value for the j th alteration (i.e., weight \times maximum score), which normalizes I_h to a value between 0 and 1. Were also calculated indices for each respective reacting patterns, I_{h1} (circulatory disturbances/inflammatory response), I_{h2} (regressive changes) and I_{h3} (progressive changes).

Table 3.1- Condition weights from histopathological alterations observed in liver samples of *S. maximus* and respective literature reference. (*) = considered for histopathological condition indices.

Reaction Pattern	Histological Alterations	Weight
Circulatory disturbances/ Inflammatory response	Melanomacrophages Infiltration/centres *	1 ^b
	Haemorrhage/Hyperaemia	1 ^a
	Lymphocytic Infiltration *	2 ^a
Regressive	Focal Necrosis *	3 ^{ab}
	Nuclear Pleomorphism	2 ^{ab}
Progressive	Fat Vacuolation of Hepatocytes *	1 ^{bc}
	Portal Vein/Bile Duct Damage	2 ^c

Source: Bernet *et al.*, (1999) ^a
 Costa *et al.*, (2009) ^b
 Zorita & Cuevas, (2013) ^c

3.6- Statistical analysis

The normality of numerical data was determined using Kolmogorov-Smirnov test, and homogeneity of variance by Levene's test. Due to invalidation of at least one test, the non-parametric Kruskal-Wallis ANOVA was applied followed by multiple comparisons test between treatments in each sampling time. Correlation analyses among indices and histological alterations were determined using the Spearman Rank test. All the statistical analyses had a significance level α set at 0.05 for all analyses. Statistical analyses were performed using the software Statistica 8.0 (Statsoft, USA).

4- Results

4.1- Liver histopathology

Fish samples from T₀ and Control (Fig. 4.1 A; B) showed regular histological liver appearance of a juvenile *S. maximus*, with normal hepatic architecture. Polygonal-shaped hepatocytes with regular-sized and well individualized nuclei located in the centre of cytoplasm and sinusoids that support and separate the basic structure were observed. The vein and bile duct were well-defined with surrounding hepatocytes (Fig. 4.1 A; B) and in some cases, erythrocytes were present within (vein). Fat vacuolation of hepatocytes (fv) represented the most frequent alteration observed and it was present in all treatments, however with different dissemination (Fig.4.3). TiO₂-NPs, BP-3 and mixture (Mix) treatments yielded higher dissemination and severity of this liver alteration at T₃, presenting larger vacuoles and the nuclei was displaced towards the cell membrane, as shown in (Fig. 4.1 C). High severity of “fv” makes hepatic tissue “whitish” which caused poor visibility of the basic hepatic structures.

Lymphocytes infiltration (Li) was characterized by a granular cytoplasm and large nucleus occupying most of the cell volume. “Li” was more representative in fish exposed to TiO₂-NPs and Mix treatments on both times (T₃ and T₇) (Fig.4.1 D). It was also quite common the presence of erythrocytes in surrounding areas or in blood vessels. Haemorrhage/hyperaemia together with lymphocyte infiltration was occasionally observed (Fig.4.2 G), in BP-3 and Mix exposure at both times (T₃ and T₇). The melanomacrophages (MMCs) appeared sometimes associated with lymphocytes in hepatic tissue and this inflammatory response was also observed (Fig.4.2 D; H; I; J) individually or in clusters next to blood vessels (bv), bile duct (bd) or in necrotic zones. The dissemination of MMCs increased considerably in liver of fish exposed to TiO₂-NPs, BP-3 and Mix treatments, however the highest dissemination was observed for Mix treatment at T₃ (Fig. 4.3). In some cases, see (Fig.4.2; J), MMCs were surrounded by a layer of connective tissue.

Nuclear pleomorphism was observed occasionally (Fig.4.1 E) and mainly in livers exposed to BP-3 at both sampling times. This alteration was observed next to blood vessels (bv) and was characterized by a visible size change in either hepatocytes or nuclei and the affected cells also displayed an enlarged and occasionally misshapen nucleus. It was also commonly accompanied by disorganization of the liver parenchyma and swelling of endothelium wall and sinusoids as seen in (Fig.4.1 E). Focal necrosis was observed along with other indicators of this issue, like pyknotic nuclei cells, lymphocytic infiltration, erythrocytes and a remarkable “white space” in hepatic structure. (Fig.4.1 D; F; and Fig.4.2 I; F). This progressive alteration was mainly observed in samples exposed to TiO₂-NPs in both T₃ and T₇ and BP-3 at T₃. In spite of those occurrences, in general, the focal necrosis did not exhibited a high rate of variability (Fig.4.3).

Additionally, but only in some individuals in Mix treatment, alterations in bile duct were reported, especially at T₇. In (Fig.4.2 H and J) are presented concentric periductal fibrosis alteration in bile duct with MMCs in surrounding areas. This alteration is considered a non-specific alteration, also known as “onion skin”, and it is characterized by inflammation (presence of MMCs). It was also observed (Fig.4.2 H) necrosis and lymphocytic cells within the bile duct structure. Also, in Mix treatment at T₇, hyalinization was observed closely to bile duct, which was characterized by lymphocytic infiltration in a homogeneous tissue with no structure. At least, no metal deposits were found in liver tissue exposed to TiO₂-NPs and Mix, through microscope observation, using the NR staining (Fig.4.2 K).

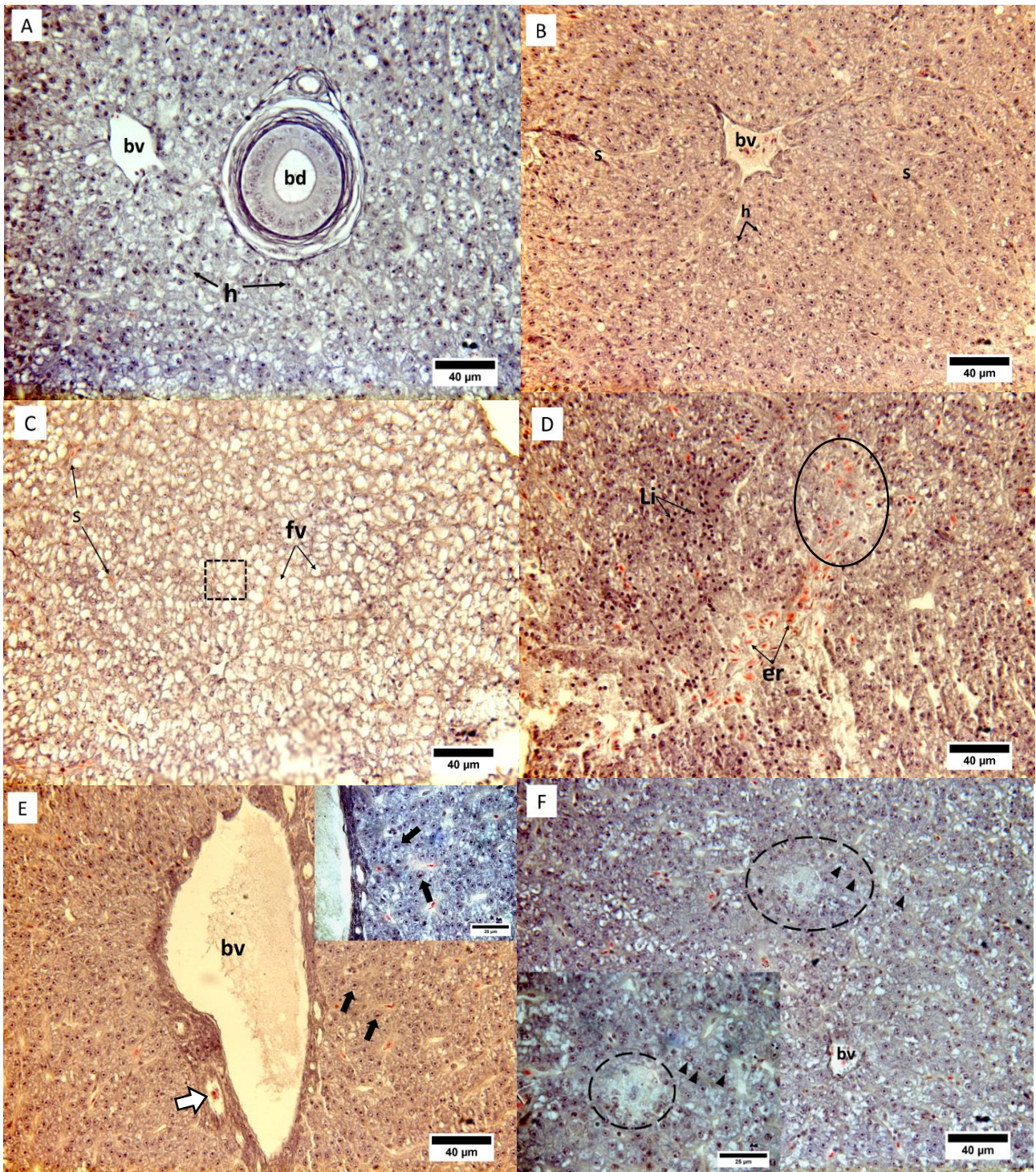


Figure 4.1- Micrographs of liver sections of *S. maximus* (H&E stain). (A and B) Normal section of a T_0 and Control fish collected in T_3 , showing normal structure of hepatic tissue with polygonal-shaped hepatocytes (h), well-defined blood vessels with epithelium membrane (bv) and bile duct (db) and sinusoids (s) supporting the structure. (C) Severe fat vacuolation of hepatocytes (fv) showing large vacuoles and nuclei displaced towards the cell membrane (square). Sinusoids (s) with erythrocytes. Higher levels of severity in livers exposed in T_3 . (D) Lymphocytes infiltration (Li) next to erythrocytes (er), the circle area displays focal necrosis and in surroundings the cell wall is identifiable. (E) Nuclear pleomorphism of cells (arrows) next to blood vessel (bv) and swelling of blood vessel wall membrane in samples exposed to BP-3, note the change in size of hepatocytes (inset). (F) Focal necrosis (circles), note the pale tone in that focal area and the pyknotic nuclei (arrow head) in adjacent cells; Inset shows in detail this alteration. Mainly appears in livers exposed to TiO_2 -NPs in T_3 .

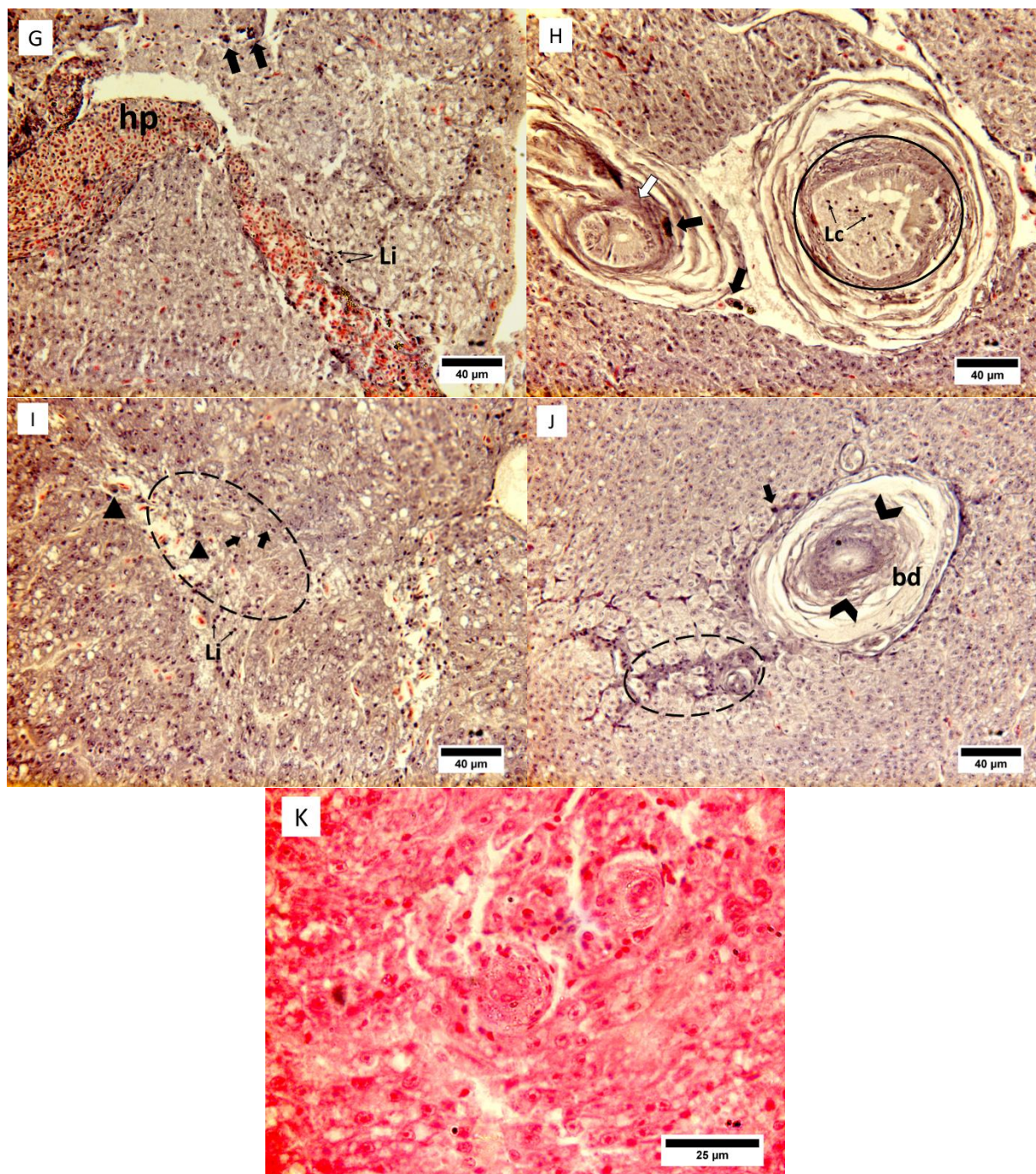


Figure 4.2- (cont.) Micrographs of liver sections of *S. maximus* (H&E stain). (G) hyperaemia (hp) in blood vessels, showing the concentration and accumulation of erythrocytes in that area. Only appears one time in Mix exposure at T₃. Note the lymphocytic infiltration (Li) and MMCs are also identified (arrows). (H) Concentric periductal fibrosis (white arrow) with some MMCs (black arrows) are present in Mix treatment at T₇. The circle area indicates necrosis of half of bile duct epithelium, note the lymphocytes cells (Lc) within necrotic area helping to digest this necrotic tissue. (I) Necrosis was seen mostly in TiO₂-NPs. The circle area indicates focal necrosis, with some indicators associated; lymphocytic infiltration (Li), MMCs (arrow head) next to erythrocytes, pyknotic nuclei cells (arrow) within necrotic area. (J) Bile duct concentric periductal fibrosis (bd), note the loss of wall membrane consistency that surround inside cells (arrow head), moderate MMCs surrounded by a layer of connective tissue (circle) very closely to bile duct, macrophage cell (arrow) surrounding bd. (K) No metal deposits were found through hepatic sections exposed to TiO₂-NPs and Mix treatments. NR stain and microscope observation.

4.2- Liver histopathological condition indices – semi-quantitative approach

Through microscope observation, were registered at least 7 types of alterations, namely:

- Melanomacrophages Infiltration/centres
- Haemorrhage/Hyperaemia *
- Lymphocytic Infiltration
- Focal Necrosis
- Nuclear Pleomorphism *
- Portal Vein/Bile Duct Damage *
- Fat Vacuolation of Hepatocytes

Among those alterations, three of them were sporadically observed (*), associated with a low frequency of observation (less than 4/5 times), or the probability to be an artefact was too high. Those alterations were inordinately distributed among the treatments and no pattern corresponding with alterations and treatments was found. Therefore, only four alterations were considered as main alterations for histopathological condition indices, namely, melanomacrophages infiltration/centres, lymphocytic infiltration, focal necrosis and fat vacuolation of hepatocytes.

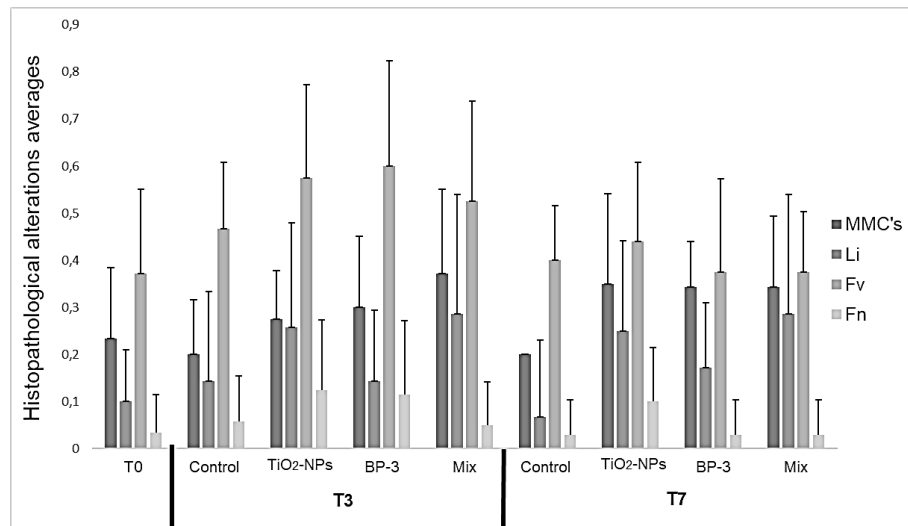


Figure 4.3- Comparison of the average between histopathological alterations in T₀ and all treatments (Control, TiO₂-NPs, BP-3 and Mixture) exposed for 3 (T₃) and 7 (T₇) days. Error bars indicate the standard deviation; MMC's: Melanomacrophages centers; Li: Lymphocytic infiltration; Fv: Fat vacuolation of hepatocytes; Fn: Focal necrosis.

As said before, the most common and frequent alterations observed as a response to contaminants in hepatic tissues were fat vacuolation (fv), melanomacrophages centres (MMCs), lymphocytes infiltration (Li) and focal necrosis (Fn), the last with less attendance. In Fig.4.3, display the variance of histopathological mean of those alterations in T₀ (immediately excised before injection groups) and throughout the groups exposed to contaminants (TiO₂-NPs, BP-3 and Mix) in both exposure times (T₃ and T₇). In general, it reveals a slight increment of alterations associated with those compounds exposure when compared with T₀ and Control samples. The “fv” and “Fn” histopathological variances suggested a decrease throughout time exposure. The histopathological variances of inflammatory responses (MMCs and “Li”) suggested a similar pattern throughout time exposure, with slight variation.

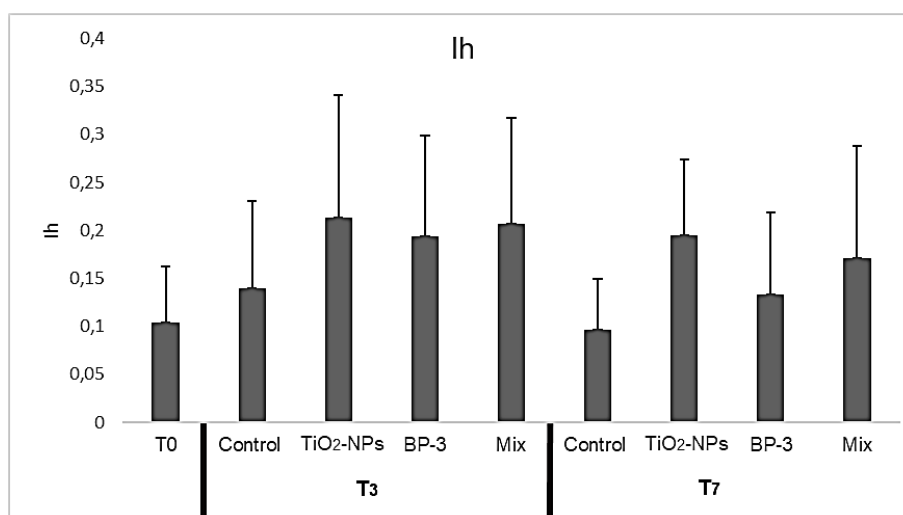


Figure 4.4- Average of histopathological condition indice (I_h) and comparison among Control, TiO₂-NPs, BP-3 and mixture treatments for 3 (T₃) and 7 (T₇) days. Error bars indicate standard deviation. (T₀: livers without treatment; Control: NaCl + DMSO; TiO₂-NPs: 3.0 µg /g of fish; BP-3: 3.0 µg /g of fish; Mix: 3.0 µg TiO₂-NPs + 3.0 µg BP-3 /g of fish).

All UV-filters treatments caused an increase in the global histopathological condition indice (I_h) compared to control group (C) and T₀ livers (Fig. 4.4). Fish exposed for 3 days (T₃) to TiO₂-NPs revealed the higher histopathological condition indice (I_h) among all treatments. However, no statistically significant differences were identified between treatments (Kruskal-Wallis ANOVA, $p > 0.05$).

Average histopathological indices score of circulatory disturbances/inflammatory response (I_{h1}), regressive changes (I_{h2}) and progressive changes (I_{h3}) are presented in Fig.4.5. In general, the I_{h1} indice showed a similar variation as the global histopathological indice, despite the Mix treatment in both exposure times (T_3 and T_7), yielding higher circulatory disturbances/inflammatory response. Therefore, the livers exposed to TiO_2 -NPs in both times of exposure showed the high score for both I_{h2} and I_{h3} . In the regressive changes (I_{h2}), at T_7 , the livers exposed to BP-3 and Mix were similar to T_0 and respective Control. Relatively to progressive alteration (I_{h3}), the exposure of TiO_2 -NPs at T_3 represented the high value, but the variance was very similar to the other treatments. However, no significant differences were indicated by Kruskal-Wallis ANOVA test ($p>0.05$). In spite of no observed differences among treatments, the results indicate a decreasing trend of average variables with the increase of time exposure (from T_3 to T_7), least in I_{h1} wherein the average variances were similar in both exposure times (T_3 and T_7).

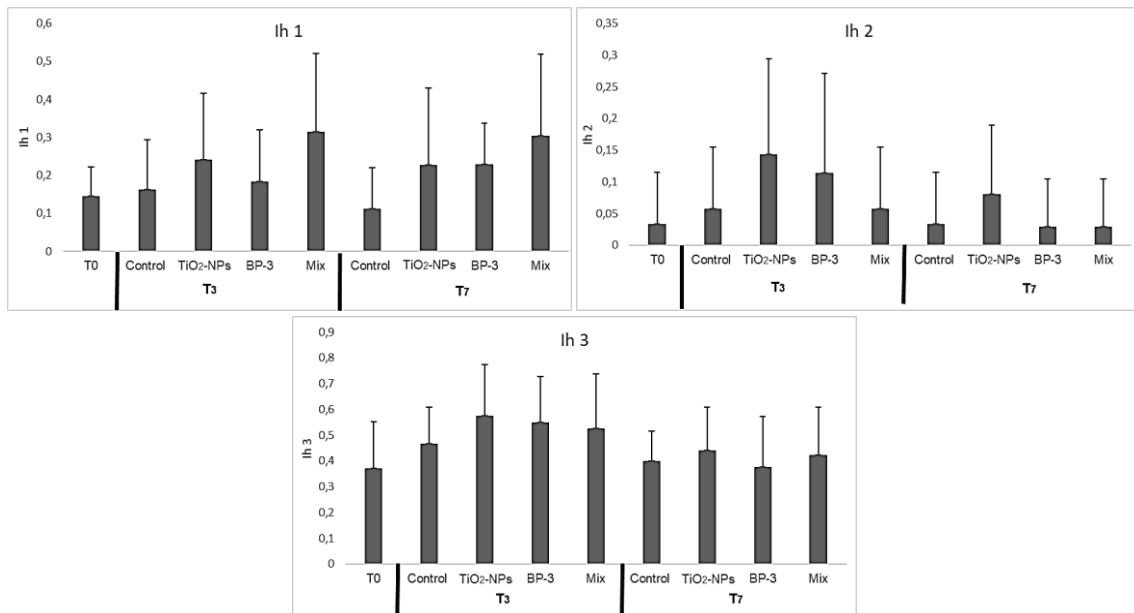


Figure 4.5- Average of each reaction pattern histopathological condition indices, and comparison among Control, TiO_2 -NPs, BP-3 and mixture treatments for 3 (T_3) and 7 (T_7) days. Histopathological condition indice circulatory disturbances/inflammatory response pattern (I_{h1}); Histopathological condition indice regressive response pattern (I_{h2}); Histopathological condition indice progressive response pattern (I_{h3}). Error bars indicate standard deviation. (T_0 : livers without treatment; Control: NaCl + DMSO; TiO_2 -NPs: 3.0 μg /g of fish; BP-3: 3.0 μg /g of fish; Mix: 3.0 μg TiO_2 -NPs + 3.0 μg BP-3 /g of fish).

4.3- Spearman *R* correlation analyses

According to the results of Spearman *R* test, the matrix in table 2, display significant correlations among indices and alterations. The highest correlation between indices was between I_h and I_{h1} ($R=0.81$, $p<0.05$) and between I_h and I_{h3} ($R=0.52$, $p<0.05$). This indicates that inflammatory/circulatory and progressive alterations were significant for the increment of the global histopathological indice. The highest correlation between alteration and the global indice was the correlation between “Li” and I_h ($R=0.77$, $p<0.05$), corroborating the observation described previously. Regarding the circulatory/inflammatory reacting pattern (I_{h1}), the “Li” was the most significant alteration ($R=0.90$, $p<0.05$) to increase this indice. Significant correlations were determined between “MMCs” and “Li” ($R=0.51$, $p<0.05$) and between “fv” and “Fn” ($R=0.29$, $p<0.05$).

Table 4.1- Spearman *R* correlations test results. Significant correlations ($p<0.05$) are presented in red.

Spearman R	I_{h1}	I_{h2}	I_{h3}	I_h	MMCs	Li	Fn	fv
I_{h1}	-	0,261	-0,015	0,809	0,484	0,907	0,241	-0,058
I_{h2}	0,261	-	0,303	0,414	0,096	0,205	0,530	0,334
I_{h3}	-0,015	0,303	-	0,519	0,249	0,097	0,276	1,000
I_h	0,809	0,414	0,519	-	0,554	0,770	0,412	0,410
MMCs	0,484	0,096	0,249	0,554	-	0,513	-0,051	0,188
Li	0,907	0,205	0,097	0,770	0,513	-	0,217	0,008
Fn	0,241	0,530	0,276	0,412	-0,051	0,217	-	0,293
fv	-0,058	0,334	1,000	0,410	0,188	0,008	0,293	-

5- Discussion

In this present Thesis, *S. maximus* fishes were exposed to environmentally-relevant concentrations of TiO₂-NPs and BP-3 compounds and their mixture, through intraperitoneal injection, during 3 and 7 days (T₃ and T₇, respectively). The liver's main role is the biotransformation and excretion of xenobiotics, which suggests that this organ is one of the target organs for environmental contamination (Hameed & Al-Azawi, 2016). Intraperitoneal injection is considered similar to oral administration, and the substances administered into mesenteric vessels drain into the portal vein and pass through the liver before reaching the systemic circulation, so the substances may suffer hepatic metabolism (Tuner *et al.*, 2011). In an attempt to predict the biological effects of two UV-filters compounds in marine fish, namely *S. maximus*, this study simulated, through intraperitoneal injection, the ingestion of an inorganic and an organic sunscreen UV-filter in environmentally-relevant concentrations. It's important to mention that, up to now, some studies have been conducted about the individual harmful effects of some emerging pollutants presented in sun care products but as far as our knowledge goes, no study was performed with mixtures of these compounds. Furthermore, few studies were done using fish as a model organism, and even fewer using fish histopathology as a biomarker analyses tool.

The histopathological characteristics of the livers from T₀ reflected the normal appearance of livers of juvenile farmed turbot (Fig.4.1 A and B) (Saraiva *et al.*, 2016). Having in mind the *I_h*, an apparent increase of the histopathological alterations was found between T₀ and control and the other treatments at both exposure times (T₃ and T₇), in spite of no observed significant statistically alterations. This trend may suggest that the hypothetical ingestion of those UV-filters compounds by *S. maximus*, could lead to slight hepatic physiological alterations. In general, the observed effects of the compounds in hepatic tissue were similar among them, however some of the alterations were specific for each compound. BP-3 treatments revealed nuclear pleomorphism while concentric periductal fibrosis was found in the Mix. The present results also suggested that TiO₂-NPs and Mix treatments yielded more severity of the hepatic alterations throughout time than BP-3. Lastly, no TiO₂-NPs deposits were found accumulated in hepatic tissue.

Moderate inflammatory response of liver induced by sunscreen UV-filters

According to the present findings, both (exposed and unexposed livers to the UV-filters) reported inflammatory responses. Nevertheless, and as previously mentioned, this response mechanism increased in livers of fish exposed to the UV-filters and their mixture. The correlation between *I_h* and *I_{h1}* was the highest, demonstrating that inflammatory/circulatory responses were fundamental for the constitution of the global histopathological condition indice (*I_h*).

In detail, the I_{h1} (Fig.4.5), showed that TiO₂-NPs and Mix treatment, in comparison to T₀ and Control treatments, generated the highest values after 3 days of exposure, suggesting that the interaction of TiO₂-NPs and BP-3 (Mix) could increase the severity of the inflammatory responses. Hobbie *et al.*, (2012) and Wettere *et al.*, (2014) suggest inflammatory response in fish liver after exposure to nitrosamine and although this response was the higher response in the UV-filters treatments, this kind of alteration is considered non-specific to these compounds (UV-filters), once it was observed in another contaminant exposure.

Findings about the TiO₂-NPs behaviour when in contact with internal organs, like liver, suggested, that different concentrations of these NPs induced toxic effects. The TiO₂-NPs immune toxic effects were tested by Jovanović *et al.*, (2010), by exposing fish (*Pimephales promelas*) to a near-environmental concentration (0.1 µg/mL) of TiO₂-NPs, reporting significant changes in the innate immune function at the levels of gene expression and cellular function. Indeed, the present results (chapter 4), suggested an increase of the infiltration of defensive cells (i.e. MMCs and lymphocytes) in liver after TiO₂-NPs injection with 3.0 µg of TiO₂-NPs /g of fish (Fig. 4.5). Federici *et al.* (2007) and Smith *et al.* (2007) reported that the livers of rainbow trout exposed to 0.1; 0.5; 1.0 mg/L of TiO₂-NPs and 0.1; 0.25; 0.5 mg/L of single walled carbon nanotubes (SWCNT), respectively, caused condensed nuclear bodies, minor fatty change and that the nanotubes surface facilitated a systemic inflammatory effect via blood. Indulekha & Thomas, (2016) revealed that after 72h of fish exposure to TiO₂-NPs at a concentration of 200 mg/L, also resulted in haemorrhage and vacuolization of hepatocytes. Moreover, after 96h of exposure pyknotic nuclei, focal necrosis, narrowing of sinusoids, irregular shaped nucleus and cytoplasmic degeneration were found. The histopathological effects observed by Indulekha & Thomas, (2016) in *Rasbora dandia* were the most similar to the results found in the present study, such as focal necrosis, pyknotic nuclei and haemorrhage (see Fig.4.1 and 4.2), however, concentrations were not comparable. Haemorrhage/hyperaemia was also associated with inflammation and was observed in Mix treatment at 73h (T₃) of exposure (Fig.4.2 G). The findings about mammals exposure to TiO₂-NPs also reported liver inflammatory response in mouse for concentrations of 10 and 50 mg/kg TiO₂-NPs administered via intragastric (Cui *et al.*, 2010) which is 3.3 and 17 times (respectively) superior than the concentrations of this study. Inflammation was also reported in rats, but in this case associated with respiratory injuries (Warheit *et al.*, 2007). Thus, the results of this study are in agreement with previous studies, regarding the occurrence of inflammation processes in fish liver due to exposition of TiO₂-NPs.

But on the contrary, similar doses of TiO₂-NPs (1.3 mg TiO₂-NPs /kg body weight), intravenously injected, induced limited effects on renal function and oxidative stress in blood of rainbow trout (Scown *et al.*, 2009). Ramsden *et al.*, (2009) indicated that 10 and 100 mg/kg TiO₂-NPs induced no alteration of the haematological parameters and that TiO₂-NPs was not a potent ionoregulatory toxic. These findings suggest a higher susceptibility of liver relative to kidney and blood, which reinforces its role in the detoxification of contaminants.

The information regarding the effects of BP-3 in fish, and particularly in liver of marine fish is still limited. Nevertheless, Díaz-Cruz *et al.*, (2008) suggested that BP-3 was able to be transformed into different metabolites namely, benzophenone-1 (BP-1) and benzophenone-2 (BP-2) and Blüthgen *et al.*, (2012) revealed that BP-3 is metabolized into BP-1 in adult zebrafish. The same authors found that concentrations of 84 µg/L BP-3 lead to gene alteration involved in steroidogenesis and hormonal pathways. Molins-Delgado *et al.*, (2018) found that BP-3 can accumulate in fish liver. According to Chen *et al.*, (2018), the major ecotoxicological concern of BP-3 is the endocrine disrupting capabilities, and in their research, the survival and growth of juvenile clown fish were not affected by 1 000 ng BP-3/g food. The present findings are the first data regarding the potential toxic effects of BP-3 in the liver of a marine fish, supporting the idea that BP-3 could trigger inflammatory responses in the liver, mainly through the increase of MMCs (please see Fig.4.3). Zorita & Cuevas, (2014) and Wolf *et al.*, (2018) stated that the intensity and prevalence of MMCs can be used as a biomarker for environmental pollution through intensity and prevalence of MMCs.

Coronado *et al.*, (2008) showed that exposure to concentrations of 620–749 µg/L BP-3 induced vitellogenin production in liver of the rainbow trout and Japanese medaka (freshwater fishes). Although it cannot be directly compared, these concentrations (620–749 µg/L BP-3) were relatively higher than the ones found in environment (~20 ng/L) and consequently higher than the ones used in the present assay (3.0 µg BP-3/g fish) and the fact that BP-3 induced that enzyme production in liver cannot be related to MMCs increase showed in the present work. Other indicator that deserves attention, is the fact that nuclear pleomorphism alteration only “appears” in livers exposed to BP-3. According to Zorita & Cuevas, (2014), nuclear pleomorphism are related with the exposure to carcinogenic compounds, and in fact, BP-3 and benzophenones family belong to aromatic ketone category (Shaath, 2005). According to the IARC, benzophenones are classified in 2B class (possible carcinogenic for humans), which may also suggest that BP-3 could have potential carcinogenetic effects in fishes.

The presence of lymphocytes cells (Li) and melanomacrophages centres (MMCs) in liver tissue, mainly at TiO₂-NPs and Mix exposure, was very clear (see Fig.4.1 D and Fig.4.2 I and J) and indicate, as said before, inflammatory response. According to Zorita & Cuevas, (2014), infiltrates of lymphocytes usually occur near to blood vessels and connective tissue. Fricke *et al.*, (2012), showed that lymphocytes were accompanied by the presence of macrophages and eosinophilic granulocytes. This description was in accordance with the results of histopathological analyses obtained in the present study. Fricke *et al.*, (2012) also reveals that infiltration of lymphocytes occurred in association with healing granulomatous lesions and regenerating areas of former necrotic tissue i.e. they were associated with the capacity of regeneration. In fact, TiO₂-NPs and Mix treatment indicated higher focal necrotic issues and inflammatory responses (Fig. 4.5), therefore, there may be a link between these two alterations as suggested by Fricke *et al.*, (2012). In the context of chronic liver diseases, like hepatocellular carcinoma, the lymphocytes had an important role in the inflammatory microenvironment favouring the initiation and progression of malignancies (Mossanen & Tacke, 2013).

Slight intensification of progressive alteration (fat vacuolation of hepatocytes) due to UV-filters exposure.

The progressive alteration, fat vacuolation of hepatocytes (fv), was the alteration that had greater dissemination throughout the liver. Various degrees of severity of “fv” were seen throughout all treatments including T₀. In many cases, “fv” was considered largely homogeneous, in that it was evenly distributed throughout the hepatic tissue, but in specific cases (less frequency) occurred in focal areas, with higher concentrations of “fat” vacuoles and due to this, it could be considered variable fat vacuolation of hepatocytes. The results of *Ih3* suggested (Fig. 4.5) that this alteration had a greater response during 3-days exposure (T₃) with a tendency to decrease over time, *i.e.* in T₇ (7 days) the response was lower and most similar to Control samples.

The turbot liver works as an organ of fat storage (lipid deposits), and this is essentially associated with the needs for fish growth in that stage (juvenile), and, because the fish source was aquaculture, the nutritional feed conditions were also associated, increasing the fat deposits in fish liver (Zorita & Cuevas, 2013; Saraiva *et al.*, 2016). This alteration was observed in fish exposed to several contaminants, for instance, Costa *et al.*, (2009); Costa *et al.*, (2011) and Martins *et al.*, (2015), observed this alteration in *S. senegalensis* liver, exposed to metallic elements (As, Cd, Cr, Cu, Ni, Pb and Zn), and to organic compounds (PAHs, PCBs and DDTs), which indicates that this alteration is not specific to a particular contaminant. On the contrary, Federici *et al.*, (2007) and Hao *et al.*, (2009) both reported minor fatty change due to exposition to TiO₂-NPs and Indulekha & Thomas, (2016) also indicated this alteration due to TiO₂-NPs exposure. In fact, Liu *et al.*, (2015) suggested that BP-3 (and other benzophenones forms/states), exposed in *Carassius auratus* liver in doses of 0.48 and 4.76 mg BP-3/L, leads to oxidative stress caused by ROS production and antioxidant responses and, at the same conditions, reported histopathological alterations in liver, such as vacuolar degeneration changes and focal necrosis. The present histopathological finds are in accordance with those histopathological alterations previously reported, where were reported focal necrosis and fat vacuolation of hepatocytes (Fig. 4.3 and Fig. 4.5).

Assessment of TiO₂-NPs deposits in fish liver

Relatively to the assessment of NPs accumulation in hepatic tissue, the results were not clear, and the method (NR satining) used for this assessment do not reveal NP accumulation on liver. Several authors had found TiO₂-NPs accumulated in fish internal organs, like liver. Specifically, Ramsden *et al.*, (2009) determined that trout showed measurable Ti accumulation (levels between 1.0 - 5.0 nmol Ti/g) in liver during dietary exposure of (10 and 100 mg/kg) TiO₂-NPs.

This NPs concentration range is one to two order of magnitude higher than the concentration used in the present assay (3.0 $\mu\text{g TiO}_2\text{-NPs/g fish}$), which suggested that environmentally-relevant NPs concentrations (used in the present research) may not contribute for the accumulation of $\text{TiO}_2\text{-NPs}$ in liver of *S. maximus*. Even so, Vignardi *et al.*, (2014) also suggest $\text{TiO}_2\text{-NPs}$ accumulation in liver (no measurable concentration data) and Scown *et al.*, (2009), reported concentrations between 0.2 - 1.1 $\mu\text{g Ti/g}$ in rainbow trout liver after high doses intravenously injection of 1.3 mg $\text{TiO}_2\text{-NPs/kg}$ body weight.

Other issue associated with the accumulation of $\text{TiO}_2\text{-NPs}$ was the generation and accumulation of ROS that can induce oxidative DNA damage in cells (Hou *et al.*, 2018). An increase in generation of ROS can occur due to excess accumulation of toxicants (Ratn *et al.*, 2018). Due to the photocatalytic activity of $\text{TiO}_2\text{-NPs}$, when exposed to UV radiation or visible light, ROS are generated (Xiong *et al.*, 2011). However, and according to Handy *et al.*, (2008), the generation of ROS associated with inflammation, raise concerns about the risk of immunotoxicity of $\text{TiO}_2\text{-NPs}$. Although it was not possible to prove the existence of nanoparticles in liver, the inflammatory response found in microscopic liver observations may suggest that ROS were generated due to the $\text{TiO}_2\text{-NPs}$ and Mix exposure. Nevertheless, other nanoparticle observation techniques, such as Transmission Electron Microscopy (TEM), and the quantification of biochemical traits could be applied in order to confirm the represent results.

6. Conclusions

To conclude, this study showed relevant results about UV-filters toxicity in *S. maximus* liver tissue. Intraperitoneal injection of environmentally-relevant concentrations of TiO₂-NPs and BP-3 induced slight histopathological alterations on *S. maximus* liver and were mainly related with the immune and inflammatory system, however no significant differences were found among these three treatments or with control animals. As such, we may conclude that environmentally-relevant concentrations of two model UV-filters compounds presents low potential risk to *S. maximus*. The mixture (TiO₂-NPs + BP-3) effects of this both UV-filters were mainly similar to the TiO₂-NPs exposure effects, which suggest that TiO₂-NPs was the “driving force” of potential toxic effects on fish liver. Despite the lack of evidence from this study about the accumulation of nanoparticles in the liver, this hypothesis cannot be discarded, and the use of other observation techniques such as TEM would provide more accurate results.

As future perspectives, the need for more research studies about the toxicologic effects of these and other UV-filters is essential, principally the evaluation of the effects of the mixture of different UV-filters compounds. Due to the dynamic functioning of marine ecosystems, the potential toxicologic effects of interaction/kinetics of the various contaminants within the ecosystem network could be amplified, hence the need for further studies. It is also important to create solutions such as the production of new sunscreen formulations that include biodegradable ingredients, like it is suggested by new research's on plant compounds that are capable of absorbing UV radiation and reach high SPF values.

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