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# Avoidance behaviour and toxicological impact of sunscreens in the teleost *Chelon auratus*

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## Abstract

There is increasing evidence that sunscreen, more specifically the organic ultra-violet filters (O-UVFs), are toxic for aquatic organisms. In the present study, we simulated an environmental sunscreen exposure on the teleost fish, *Chelon auratus*. The first objective was to assess their spatial avoidance of sunscreen products. Our results showed that the fish did not avoid the contaminated area. Therefore, the second objective was to evaluate the toxicological impacts of such pollutants. At the individual level, O-UVFs increased the hepatosomatic index which suggests pathological alterations of the liver. At the cellular level, a significant increase of malondialdehyde was measured in the muscle of fish exposed to O-UVFs which suggests a failure of antioxidant defences and/or an excess

of reactive oxygen species. Although our study highlighted some of the toxicological effects due to O-UVFs contamination, further investigations are needed for a better understanding of the impact of these contaminants upon marine teleosts.

**Keywords: Sunscreen, Avoidance behaviour, Oxidative stress, Hepatosomatic index, Fulton index, Specific growth rate**

## **1. Introduction**

Coastal zones are areas of rich biodiversity and provide natural resources to human societies. However, they are strongly affected by anthropogenic pressures such as the input of contaminants (IPCC, 2021). Among these contaminants, sunscreen products are emerging pollutants that can be found in the water column of sunbathing areas from a few  $\text{ng.L}^{-1}$  (Magi et al., 2012; Labille et al., 2020) to several  $\mu\text{g.L}^{-1}$  (regarding the octocrylene: Langford and Thomas, 2008 observed  $7.3 \mu\text{g.L}^{-1}$  of OC on a beach in Oslofjord (Norway); Sánchez Rodríguez et al., 2015 recorded  $1.3 \mu\text{g.L}^{-1}$  of OC on a beach in Gran Canaria (Canary Islands, Spain); Bratkovics et al., 2015 measured  $3.7 \mu\text{g.L}^{-1}$  of OC on a beach located in South Carolina (USA)). These concentrations mainly depend on human recreational activities, which increase during the summer season, as well as on the presence of wastewater effluents (Bachelot et al., 2012; Sánchez Rodríguez et al., 2015). Moreover, several studies highlighted a diurnal variation of O-UVFs concentration in the sunbathing areas due to the human recreational activities pressure (Picot-Groz et al., 2018 and Sankoda et al., 2015). Recently, the scientific community has shown an increasing interest in these sunscreen products, given the effect their active ingredients – the organic ultra-violet filters (O-UVFs) – have on the aquatic biota (Lozano et al., 2020; Carve et al., 2021). Indeed, their toxicity has been demonstrated upon on the reproduction (e.g. in the fathead minnows *Pimephales promelas*, Christen et al., 2011), the development (e.g in the crustacean *Siriella armata*, the sea-urchin *Paracentrotus lividus*, the bivalve *Mytilus galloprovincialis*, Paredes et al., 2014; in zebrafish *Danio rerio*, Balázs et al., 2016; in the planktonic crustacean *Daphnia magna*, Lambert et al., 2021) and the level of oxidative stress in a wide range of organisms from protozoan *Tetrahymena thermophila* (Gao et al., 2013) to the

loggerhead sea turtle *Caretta caretta* (Cocci et al., 2020). However, most of these studies were conducted using exposure protocols that consider only a single molecule, *i.e.* without integrating potential cocktail effects between various O-UVFs or between various O-UVFs and excipients such as emollients, emulsifiers and perfumes (Park et al., 2017; He et al., 2019; Carve et al., 2021). Furthermore, most authors used higher concentrations than those measured in seawater (Fivenson et al., 2021) and, frequently, with an acute exposure mode (Carve et al., 2021) of 24–96h (Kim et al., 2011; Sun et al., 2016; Almeida et al., 2019) while the presence of O-UVFs in the water column of recreational activities can last the entire summer season (Sankoda et al., 2015; Kim et al., 2017).

Regarding the biological models used to investigate O-UVFs toxicity, a review of literature (Carve et al., 2021) showed an imbalance in favour of freshwater organisms to the detriment of marine ones. Among the small corpus of literature focusing on marine organisms, corals are studied (Lozano et al., 2020) since O-UVFs may contribute to their bleaching by triggering the expulsion of their symbiotic algae, zooxanthellae (Downs et al., 2016; He et al., 2019). Other species such as mussels (Paredes et al., 2014; Giraldo et al., 2017; Bordalo et al., 2020; Falfushynska et al., 2021) and sea urchins (Paredes et al., 2014; Torres et al., 2016; Giraldo et al., 2017; Corinaldesi et al., 2017; González et al., 2022) have also received attention due to their propensity to accumulate O-UVFs (Picot Groz et al., 2014; Rocha et al., 2018) and their role as sentinels of the aquatic environment (Beyer et al., 2017; Parra-Luna et al., 2020). However, few studies focused on the effects of O-UVFs on marine teleosts (Table 2 in Carve et al., 2021).

Regarding the effects of O-UVFs on behaviour, to our knowledge, few studies have investigated the detection and avoidance of sunscreen products by organisms. Among these studies, no avoidance response has been recorded for flatworms *Convolutriloba macropyga* (McCoshum et al., 2016), in contrast to the findings for shrimp *Palaemon varians* (Araújo et al., 2020). Avoidance of the stress source could, however, prevent physiological impacts, according to several authors (Claireaux et al.,

2018; Moreira-Santos et al., 2019; Alcívar et al., 2021). This behaviour is of great interest when estimating the ecotoxicity of O-UVFs on organisms.

Thus, since most of the studies were conducted on freshwater organisms, we investigated the effects of O-UVFS on a marine teleost species, the golden gray mullet *Chelon auratus*. This species was chosen due to its abundance in littoral habitats (e.g. estuaries, saltmarshes, Laffaille et al., 1998) along the North-Western Atlantic coast and in the Mediterranean Sea and because of its ecological benefit: *C. auratus* are considered ecosystem engineers since they are major actors in the trophic web through the transfer of organic matter between habitats (Lebreton et al., 2011).

Moreover, since most of the studies exposed organisms to a single molecule using an acute exposure mode, we conducted two experiments, in which fish were exposed more environmentally realistic sunscreen contamination. Regarding the lack of studies exploring the link between O-UVFs and fish behaviour, the first experiment was aimed at evaluating the detection capacity and the avoidance behaviour when fish were exposed to O-UVFs. It was conducted using a free-moving experimental set-up containing two different water masses (i.e. clean versus contaminated seawater). The second experiment was designed to describe the toxicological effects following long term exposure (as observed in tourism areas during the summer season). To do so, two levels of organisation were considered: the sub-individual and the individual. At the sub-individual level, oxidative damages were assessed through lipid and protein oxidation in target organs and tissues: (i) the liver, regarding the detoxification function of this organ; (ii) the gills, which are in contact with the external environment and potentially the first target organ to be impacted; and finally (iii) the muscle, since this organ is in relation to locomotor performance, which is primordial for activities such as exploring habitat, foraging, escaping and mating. In parallel to these sub-individual markers, individual indicators of general health status (i.e. the fulton index FI, the specific growth rate SGR and the hepatosomatic index HSI) were measured, considering their ecological pertinence (Amiard and Amiard-Triquet,

2017). Furthermore, the FI, the SGR and the HSI indices were considered regarding the fact that they could be modulated by organic pollutants (Kerambrun et al., 2012a,b; Lucas et al., 2021).

## 2. Materials and Methods

### 2.1. Fish maintenance

Juvenile golden gray mullets were captured in l'Houmeau salt marshes (46°12'14"N 1°11'42"W) with fyke nets during summer 2020. Then, they were transferred into 400 L indoor tanks (n = 20 individuals per tank) in our laboratory (Institut du Littoral et de l'Environnement, UMR 7266 CNRS-La Rochelle University, France). The quality of the natural and aerated seawater was maintained with an external biological and mechanical filter (Eheim professional 3 2080, Eheim, Deizisau, Germany) and a weekly 30% water change. Two months before the experiments began, the acclimation temperature was set to 20°C using a thermoregulator (TECO® Refrigeration technologies, Ravenna, Italy) with a hysteresis of  $\pm 0.5^\circ\text{C}$ . In the acclimation tank during this period, fish were exposed to a 12h light: 12h dark photoperiod cycle and fed at least three times per week with commercial food pellets (Neo Repro II, Le Gouessant, France). Nitrites and oxygen levels were daily controlled and were, respectively, below  $0.1 \text{ mg}\cdot\text{L}^{-1}$  and above 90% air saturation.

Experiments were carried out with respect to regulations of the Animal Care Committee of France (ACCF) (APAFIS#25159-2019102913067306 v7; UMR7266 LIENSs approval number: 173002).

### 2.2. Sunscreen exposure media

The sunscreen exposure media was used in both experiments and was made from two commercial sunscreens mixed in sea water. These two sunscreens were selected because, together, they contained the four major O-UVFs (*i.e.* octocrylene OC; 2-ethyl-hexyl-4-trimethoxycinnamate EHMC and Ethylhexyl Salicylate EHS) found in 101 sunscreen products used by sunbathers on a beach on the French Atlantic coast (Minimes, La Rochelle) during summer 2019 (field survey, ANR-CUTE project, unpublished results). It should be noted that both sunscreens do not contain mineral UVFs in

their formulation but only organic UVFs and excipients. The list of ingredients for the two commercial sunscreens used is provided in Table 1 of the supplementary materials. The concentration of each ingredient is not provided (according to the European regulation CE No 1223/2009 for cosmetic products, manufacturers do not have to include concentrations), but the order of appearance on the list of ingredients reveals the most used ones.

The sunscreen exposure media was developed within the framework of this experimentation and was obtained as follows: 4.8 g of each sunscreen was added to 1.2 L of 5 $\mu$ m-filtered natural seawater in a 2 L glass beaker. After 24 hours of mixing with a Magnetic-Stirrer and 2 hours of decanting in the dark at ambient temperature, the sunscreen solution was filtered, with a vacuum pump, using a glass microfiber filter with a pore size of 0.47  $\mu$ m (GF/F, Whatman, GE Healthcare, Little Chalfont, UK). This last step permitted to reduce the particulate fraction of the mixture. The obtained solution was homogeneous, with no particles and was stored in the dark at 4 °C before use.

### 2.3. First experiment: Avoidance test

#### 2.3.1. Choice tank set-up (Figure 1)

The experimental set-up was composed of two choice tanks (Loligo Systems, Tjele, Denmark: L:130 x W:40 x H:20 cm), each supplied by two 450 L-polyethylene seawater cisterns. Choice tanks allow us to investigate fish distribution within two adjacent water masses, which are maintained independently due to a stable horizontal gradient. In our experiment, the contamination of only one of the two water masses with the sunscreen exposure media will allow us to evaluate the distribution of the fish between a contaminated and an uncontaminated environment, *i.e.* their capacity of avoidance from a contaminated environment.

A choice tank is divided in three sections: (a) an upstream area (L: 57 cm x W: 40 cm) with two longitudinally and separated compartments, where a succession of honeycombs promotes a laminar flow of water before reaching the test area; (b) a test area (L: 32 cm x W: 40 cm), where the animal

can move freely between the two adjacent and laminar water masses; (c) a downstream area (L: 41 cm x W: 40 cm), which receives water from the test area before it overflows to a drain.

Each 450 L-cistern supplies one of the separate compartments of the upstream area of the choice tank using a hydraulic pump (Eheim GmbH & Co. KG.). The water temperature from each cistern is maintained at  $20.02^{\circ}\text{C} \pm 0.12^{\circ}\text{C}$  (mean  $\pm$  standard error (SE)), using a heater (500 W) coupled to a control box (AQUA MEDIC T Controller Twin). The water is sent to the choice tank through a dedicated valve, which allows the experimenter to control the water flow at  $950 \text{ L}\cdot\text{h}^{-1}$  (monitored using a flowmeter). The laminar flow and the consequent creation of two separate water masses were checked before the experiment began. In order to do so, food colouring was introduced into only one of the two cisterns to verify that it spread only to the corresponding side of the test area.

In order to determine the spatio-temporal distribution of the fish between these two environments, a GoPro Hero7 camera was positioned above the test area.

### 2.3.2. Experimental protocol

A total of 25 fish [mean weight  $\pm$  SD:  $7.35 \text{ g} \pm 2.60 \text{ g}$ ; mean total length  $\pm$  SD:  $9.44 \text{ cm} \pm 1.10 \text{ cm}$ ] were tested individually in the choice tanks in order to evaluate their avoidance behaviour. Two groups of fish were formed. The *Control* group ( $n = 14$ ) of *C. auratus* had the choice between two uncontaminated environments, while the *Sunscreen exposure* group ( $n = 11$ ) had the choice between an uncontaminated environment or an environment contaminated with the sunscreen exposure media.

In order to test their avoidance behaviour, the experimental protocol was designed as follow: *C. auratus* were starved for 24h before beginning the avoidance behaviour test. One fish was transferred from its 400 L acclimation tank to the test area. After 2 hours of acclimation, a 1<sup>st</sup> video recording event allows to quantify the time spent in each environment (side) of the test area over a 15 minute period. This measurement was accomplished using a chronometer and performed in real-time. It did not induce disturbance of the fish since the experimenter observed the videos from a



smartphone connected to the camera, using the GoPro Quik app. At this point, no contaminant was introduced into the device, and the most visited side by the fish (cumulative time greater than 7.5 minutes) before the injection of any contaminant was considered to be its preferred side, noted as PS. The time spent in the PS before the injection was noted as  $T_{PS1}$ .

After this first period of measurement, 100 mL of exposure media or uncontaminated water (depending on the fish group) was added to one of the cisterns, in order to contaminate the PS. A waiting period of 5 minutes was respected in order to ensure the mixing of the exposure media and water in the 450 L-cistern. Then, a second video recording event was made over a period of 15 minutes, in order to evaluate the time spent in the PS after injection of the contaminant (or water), noted as  $T_{PS2}$ .

*In fine*, the two video recording events (*i.e.* before and after the injection of the sunscreen exposure media or the addition of the placebo) allowed us to calculate an avoidance index (AI) as follows:

$$AI = \frac{T_{PS1}}{T_{PS2}}$$

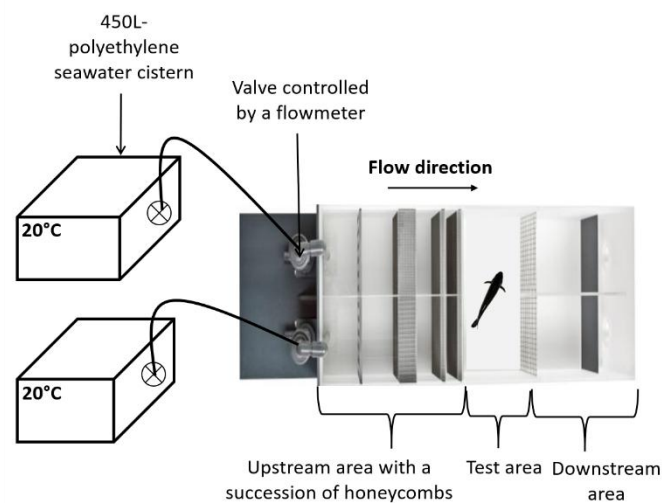
Where  $T_{PS1}$  is the time spent in minutes in the PS before the injection of the sunscreen exposure media (*i.e.* measured during the first recording video event) and  $T_{PS2}$  is the time spent in minutes in the PS after the injection of the sunscreen exposure media (*i.e.* measured during the second recording video event).

A spatial avoidance was established if the AI of fish belonging to the *Sunscreen exposure* group was significantly higher when compared to the AI of fish belonging to the *Control* group.

At the end of the video recording period, 200 mL of seawater was taken from each side of the test area for the O-UVFs extraction and placed at -20°C. Then, the fish were anaesthetised with tricaine methane sulphonate (MS-222; 0.1 g L<sup>-1</sup>, Sigma-Aldrich, St Quentin-Fallavier, France) and biometry (*i.e.* total length, specific length, width, height and weight) were taken. Each fish was then

transferred to a recovery tank with parameters (temperature, salinity, oxygen and nitrite) similar to the 400 L-acclimation tanks.

Between each avoidance choice test, the entire experimental set-up was washed three times with fresh water. Two choice tanks were used so that two fish could be tested per day and each choice tank could be used for fish belonging to the *Control* or to the *Sunscreen exposure* group, in order to avoid any experimental set-up effect.



**Figure 1: Scheme of the choice tank.** The seawater is supplied by two 450 L cisterns, using hydraulic pumps (identified by ⊗). Water circulates through several honeycombs into the upstream area and then into the test area without mixing. Finally, the water passes through the downstream area before draining out of the tank.

## 2.4. Second experiment: Effect of chronic O-UVFs contamination on general health status and oxidative stress of *C. auratus*

### 2.4.1. Fish tagging

After two months of acclimation in the 400 L- acclimation tanks, 24 fish (different from those used for the avoidance tests) were tagged with a fluorescent elastomer, so they could be identified later with a UV lamp (405 nm, Northwest Marine Technology, INC). To do so, each individual was anaesthetised with tricaine methane sulphonate (MS-222; 0.1 g L<sup>-1</sup>, Sigma-Aldrich, St Quentin-Fallavier, France). Then, the fluorescent elastomer was injected subcutaneously in front of the first

dorsal fin using a syringe. Each fish was tagged with a unique combination of colour (*i.e.* yellow or red), position and number of fluorescent tags. Once the elastomer was injected, fish were transferred directly into the exposure aquaria (described below) and were allowed to recover for four days before the exposure began.

#### 2.4.2. Set-up of the exposure aquaria

Two groups of fish were placed in 100 L-exposure aquaria after tagging: *Control* group [n = 12, mean weight  $\pm$  SD: 37.21 g  $\pm$  9.37 g; mean total length  $\pm$  SD: 16.61 cm  $\pm$  1.52 cm] and *Sunscreen exposure* group [n = 12, mean weight  $\pm$  SD: 30.89 g  $\pm$  3.96 g; mean total length  $\pm$  SD: 15.58 cm  $\pm$  1.1 cm].

During the entire experimental period, *C. auratus* were fed daily with commercial pellets (Neo Repro II, Le Gouessant, France) with an amount equal to 1.5% of their biomass. The air conditioning system of the experimental room permitted us to maintain the temperature of the aquaria at 19.91°C  $\pm$  0.05°C (mean  $\pm$  SE) for the *Control* group and 20.14°C  $\pm$  0.06°C (mean  $\pm$  SE) for the *Sunscreen exposure* group. Temperature, nitrites (0.20 mg.L<sup>-1</sup>  $\pm$  0.02 mg.L<sup>-1</sup> (mean  $\pm$  SE) for both groups) and oxygen levels (superior to 80% of air saturation) were controlled twice a day. To ensure consistent water quality, the seawater of both aquaria was renewed daily with 200 L of seawater by an overflow system, and the oxygen level was maintained by an air-pump.

#### 2.4.3. Experimental protocol

Throughout the 35 day sunscreen exposure period, the *Sunscreen exposure* group received 50 mL of the exposure media (cf. part 2.2.) daily, which was injected into the water column of the 100 L-exposure aquaria. At the same time, the *Control* group received 50 mL of seawater. The renewal of the aquaria with clean sea water (conducted 6 hours after the introduction of the exposure media) permitted us to simulate the daily variation in concentration of O-UVFs in sunbathing areas during the summer season on tourist beaches (Picot-Groz et al., 2018). This daily variation of the O-UVFs concentration was evaluated by sampling 200 mL of seawater in the water column of the *Sunscreen exposure* group aquaria on the 20<sup>th</sup> day of contamination. Seawater was sampled at 30 min, 1h30,

2h30, 3h30 and 4h30 after injection of the exposure media and then, 30 minutes after the water renewal (*i.e.* 6h30 after the addition of the contaminant). To check the absence of contamination in the 100 L-exposure aquaria of the *Control* group, 200 mL of seawater was also collected on the 20<sup>th</sup> day of contamination. In order to reduce the exposure of the fish to stress, these procedures were completed only once and were considered to be representative of the daily variation occurring throughout the 35 days of experimental exposure. The stability of this pattern was confirmed by punctually measuring O-UVFs concentrations on the 35<sup>th</sup> day of contamination (*i.e.* at 14h and 18h). Until O-UVFs extraction, the samples were stored at -20°C.

At the end of the sunscreen exposure period, no mortality was recorded.

#### 2.4.4. Measurements of indicators for general health status

Weight, height, width, total and specific length were measured the day before the introduction of individuals into their aquarium (T1) and three days after the 35 days of sunscreen exposure (T2). The liver wet weight was also measured at T2. To do so, the individuals were anaesthetised at T1 and euthanised at T2, using different concentrations of tricaine methane sulphonate (MS-222; 0.1 g L<sup>-1</sup> and 0.5 g L<sup>-1</sup>, respectively; Sigma-Aldrich, St Quentin-Fallavier, France). At T2, the liver, muscles and gills were collected from euthanatised fish and flash frozen before being placed at -80°C for further biochemical analysis of the oxidative stress (described in 2.4.5).

Based on the biometry data, different parameters were calculated for each individual:

- the specific growth rate (SGR, in % day<sup>-1</sup>):

$SGR = \frac{W_f - W_s}{t} \cdot 100$ , where  $W_f$  is the body mass of the fish (g) at T2,  $W_s$  is the body mass of the fish (g) at T1,  $t$  is the number of days between  $W_f$  and  $W_s$ .

- the Fulton Index (FI, in %) as an index of the body condition:

$FI = \left( \frac{W_f}{(L_t)^3} \right) \cdot 100$ , where  $W_f$  is the body mass of the fish (g) at T2 and  $L_t$  is the total length of the fish (cm) at T2.

- the hepatosomatic index (HSI, in %) as an index of the energy reserves:

$HSI = \frac{W_l}{W_f} \cdot 100$ , where  $W_l$  is the liver wet weight (g) at T2 and  $W_f$  is the body mass of the fish (g) at T2.

#### 2.4.5. Oxidative damage

Three days after the 35 days of sunscreen exposure (*i.e.* at T2), protein and lipid oxidation assays were performed on the samples of liver, gills and muscle from the euthanatised *C. auratus*.

##### 2.4.5.1. Sample preparation

Briefly, the organs were diluted in a phosphate buffer solution (PBS, 315 mOsmol ; pH 7.4) with a tissue weight : buffer ratio of 1 : 2. The organ was then ground with a Teflon potter to obtain an intermediate homogenate. A volume of 110  $\mu$ L of this intermediate homogenate was added to the same volume of PBS for additional grinding in order to obtain the final homogenate. After two successive centrifugations (12,500 g, 4°C) of the final homogenate, the supernatant was divided into 3 different tubes for assays of protein concentrations, as well as malondialdehyde (MDA) levels and carbonyl content.

##### 2.4.5.2. Biomarkers assessment

###### 2.4.5.2.1. Protein assay

Total protein concentrations were assessed using the BCA Protein Assay Kit (Novagen<sup>®</sup>, Germany), which contains bovine serum albumin as a standard and Cupric sulfate as a reagent. The reaction is based on the alkaline reduction of  $Cu^{2+}$  to  $Cu^{1+}$  by proteins, and the detection of the monovalent copper ions  $Cu^{1+}$ . The purple solution was measured at an absorbance of 562 nm on a microplate, using the spectrophotometer SPECTROstar<sup>Nano</sup> (BMG LABTECH).

#### 2.4.5.2.2. MDA extraction

The protocol for the MDA extraction was adapted from the Bioxytech MDA-586 kit (OxisResearch, Spain). The method was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45°C. The blue solution was measured at an absorbance of 586 nm on a microplate, using the spectrophotometer SPECTROstar<sup>Nano</sup> (BMG LABTECH). The results are presented in  $\mu\text{mole}$  of MDA/g of protein.

#### 2.4.5.2.3. Carbonyl content

The protocol that we followed was adapted from the Protein Carbonyl Colorimetric Assay Kit (Cayman chemical). The method is based on the reaction between 2,4-dinitrophenylhydrazine and protein carbonyls at ambient temperature. The yellow solution containing hydrazone-protein was measured at an absorbance of 380 nm, using the SPECTROstar<sup>Nano</sup> (BMG LABTECH). The results are presented in nmole of carbonyl/mg of protein.

### 2.5. Seawater concentrations of O-UVFs

The liquid-liquid extraction (LLE) process, which includes applying methyl tertiary-butyl ether (MTBE) to the seawater samples, was used prior to detection and quantification of O-UVFs (*i.e.* OC, EHS, EHMC) by using a gas chromatography system (Agilent HP 7890N) coupled with an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies). For the chromatography portion, helium was used as the carrier gas at  $1 \text{ mL}\cdot\text{min}^{-1}$ ; the GC column was a Restek RXi-5ms (30 m x 0.25 mm / ID x 0.25  $\mu\text{m}$ ; Bellefonte, USA). The oven temperature programme that we used was 70°C (3 min) followed by an increase of  $20^\circ\text{C}\cdot\text{min}^{-1}$  until 180°C, and a final increase of  $10^\circ\text{C}\cdot\text{min}^{-1}$  until 280°C (5 min). The mass spectrometer was operated in MRM mode with two transitions for each compound of interest. Results are expressed as  $\mu\text{g}\cdot\text{L}^{-1}$ .

### 2.6. Statistical analyses

Levene's and Bartlett's tests were used to ensure the conditions of homoscedasticity and normality of the data for each variable (*i.e.* avoidance index, HSI, FI, SGR, MDA and carbonyl content). If the conditions were met, a T-test was performed to highlight significant differences between the two groups. Otherwise, a non-parametric test was used (*i.e.* Mann–Whitney U test). The results of the tests were considered significantly different if  $P < 0.05$ . The values were expressed as mean  $\pm$  standard error (SE) of the mean.

### 3. Results

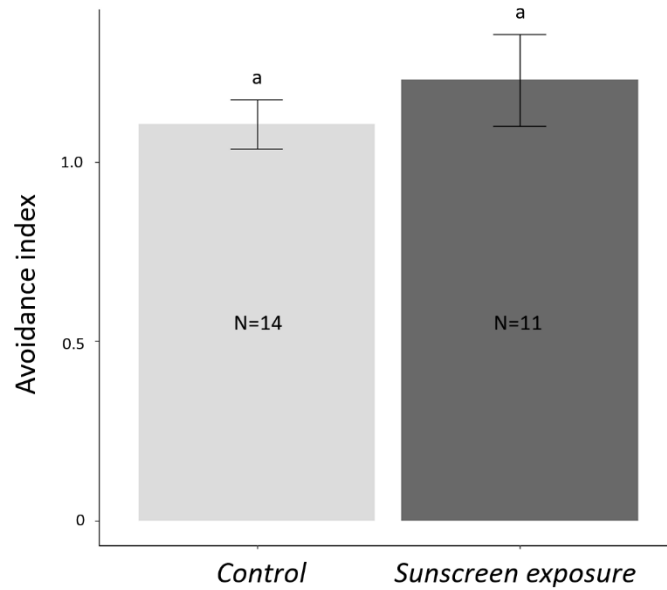
#### 3.1. First experiment: Avoidance test

##### 3.1.1. Water analysis

At the end of the video recording period, the three O-UUVs were below the limit of detection ( $< \text{LOD}$ ) in the uncontaminated side of the choice tank while, as expected, O-UUVs were found in the contaminated side. The O-UUV with the highest concentration was the OC (mean  $\pm$  SE:  $1.61 \mu\text{g.L}^{-1} \pm 0.51 \mu\text{g.L}^{-1}$ ), followed by the EHMC (mean  $\pm$  SE:  $1.36 \mu\text{g.L}^{-1} \pm 0.42 \mu\text{g.L}^{-1}$ ) and finally by the EHS (mean  $\pm$  SE:  $0.68 \mu\text{g.L}^{-1} \pm 0.20 \mu\text{g.L}^{-1}$ ). Consequently, the sum of the concentrations for the three detected O-UUVs was  $3.65 \mu\text{g.L}^{-1} \pm 1.08 \mu\text{g.L}^{-1}$  (mean  $\pm$  SE) of pollutants.

##### 3.1.2. Behavioural response

The avoidance index varied from  $1.10 \pm 0.07$  for *C. auratus* in the *Control* group to  $1.23 \pm 0.13$  for *C. auratus* in the *Sunscreen exposure* group. No statistical difference was found between these 2 groups (Mann–Whitney U test,  $W = 67$ ,  $P = 0.60$ ), demonstrating that the presence of sunscreen did not induce an avoidance behaviour in *C. auratus* (Figure 2).



**Figure 2: Avoidance index of *C. auratus* for the *Control* (light grey) and *Sunscreen exposure* (dark grey) groups.** Values represent mean  $\pm$  standard error. Identical letters above bars indicate no difference between *Control* and *Sunscreen exposure* groups ( $P < 0.05$ ).

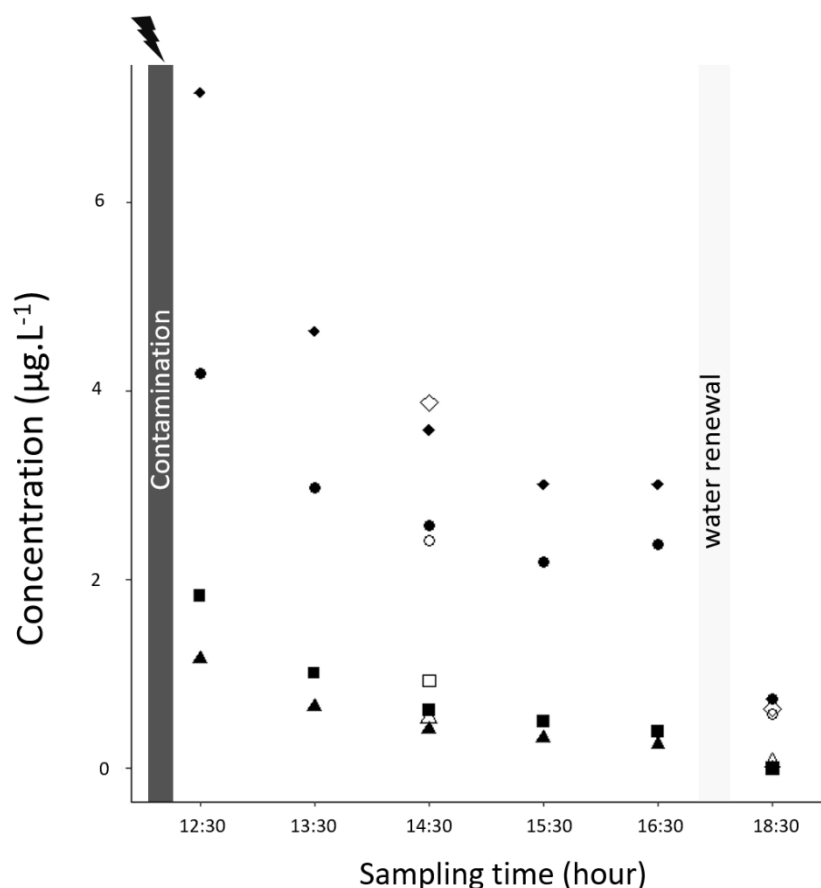
3.2. Second experiment: Effect of chronic O-UVFs contamination on general health status and oxidative stress of *C. auratus*

### 3.2.1. Water analysis

On the 20<sup>th</sup> day of contamination, the concentrations of the three O-UVFs were below the limit of detection ( $< LOD$ ) in the 100 L - exposure aquaria of the *Control* group. In the *Sunscreen exposure* group, the three O-UVFs were in the order of magnitude of a few  $\mu\text{g.L}^{-1}$  (from  $1.15 \mu\text{g.L}^{-1}$  for EHS to  $4.18 \mu\text{g.L}^{-1}$  for OC) 30 minutes after the contamination event. Furthermore, an expected daily decrease (*i.e.* in 6 hours) in O-UVFs concentration was recorded (Figure 3): OC concentrations went from  $4.18 \mu\text{g.L}^{-1}$  at 12:30 to  $2.37 \mu\text{g.L}^{-1}$  at 16:30 before water renewal and dropped to  $0.73 \mu\text{g.L}^{-1}$  after it (*i.e.* at 18:30); EHS and EMHC concentrations went, respectively, from  $1.15 \mu\text{g.L}^{-1}$  and  $1.83 \mu\text{g.L}^{-1}$  at 12:30 and  $0.25 \mu\text{g.L}^{-1}$  and  $0.39 \mu\text{g.L}^{-1}$  at 16:30 to a concentration below the limit of quantification ( $< LOQ$ ) after the water renewal. Thus, when considering the totality of O-UVFs, a decrease of 89.77% was measured after the water renewal (from  $7.17 \mu\text{g.L}^{-1}$  at 12:30 to  $0.73 \mu\text{g.L}^{-1}$  at



18:30). On the 35<sup>th</sup> day of contamination, a similar pattern of O-UVFs concentrations was observed (Figure 3): the OC decreased from 2.41  $\mu\text{g.L}^{-1}$  at 14:30 to 0.57  $\mu\text{g.L}^{-1}$  at 18:30; the EHMC decreased from 0.93  $\mu\text{g.L}^{-1}$  at 14:30 to a concentration below the LOQ at 18:30, and the EHS decreased from 0.53  $\mu\text{g.L}^{-1}$  at 14:30 to 0.06  $\mu\text{g.L}^{-1}$  at 18:30.

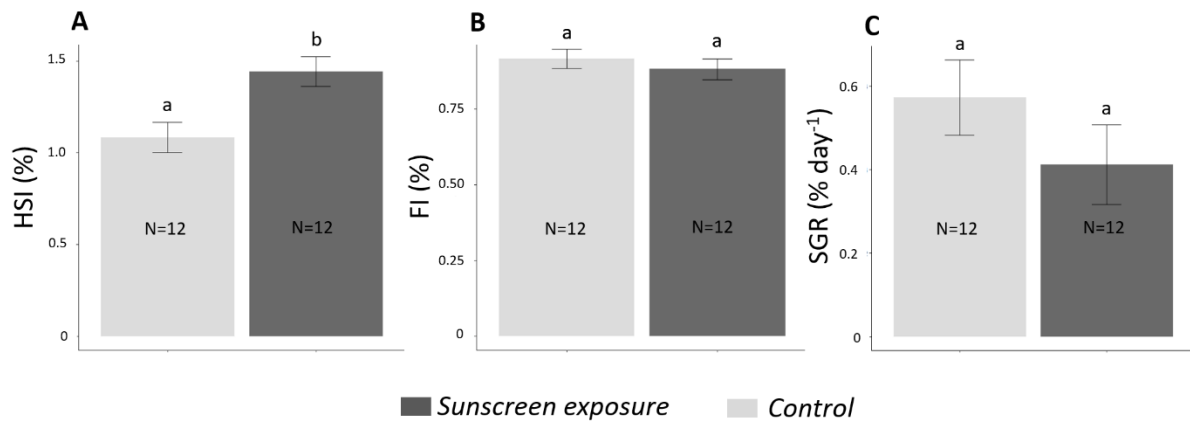


**Figure 3: Evolution of the concentration of O-UVFs ( $\mu\text{g.L}^{-1}$ ) during the days of contamination.** Each symbol corresponds to a single O-UVFs measurement. For the 20<sup>th</sup> day of contamination: ● is for octocrylene OC, ▲ is for ethylhexyl Salicylate EHS, ■ is for 2-ethyl-hexyl-4-trimethoxycinnamate EHMC and ◆ is for the total of the three precedent O-UVFs. Concentration at 14:30 and 18:30 on the 35<sup>th</sup> day of contamination were indicated with the same symbols but empty. In the 100 L-exposure aquarium of the *Control* group, O-UVFs were not detected (*i.e.* <LOD).

### 3.2.2. Indicators of general health status

HSI (Figure 4A) showed a statistical difference between the two groups of fish (Mann–Whitney U test,  $W = 25$ ,  $P = 0.007$ ). Indeed, the 35 days of exposure induced a 33% hepatosomatic index increase for the *Sunscreen exposure* group. On the other hand, the analysis of FI (Figure 4B) revealed no

statistical difference (Mann–Whitney U test,  $W = 88$ ,  $P = 0.37$ ). Regarding SGR (Figure 4C), no statistical difference (Mann–Whitney U test,  $W = 96$ ,  $P = 0.17$ ) was recorded either, despite a lower value for the group that was exposed to the sunscreen exposure media (*i.e.* from  $0.57 \pm 0.09$  for the *Control* group to  $0.41 \pm 0.10$  for the *Sunscreen exposure* group).

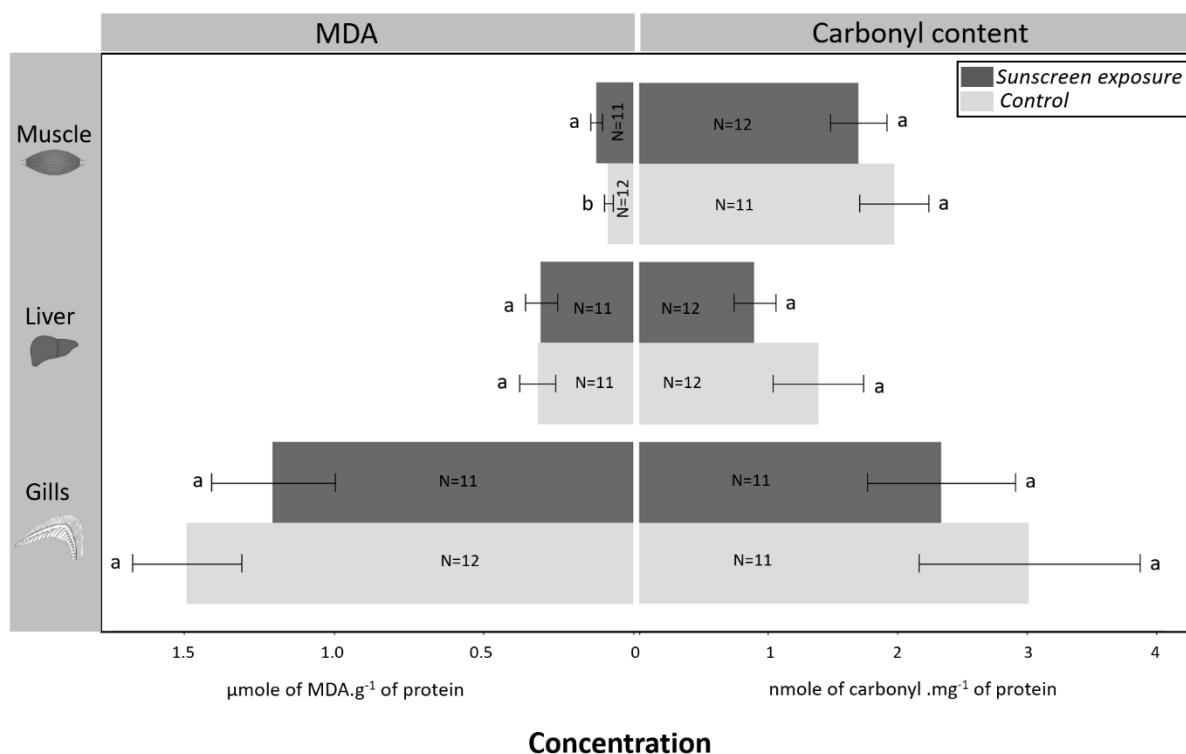


**Figure 4: Hepatosomatic index (HSI), Fulton index (FI) and specific growth rate of *C. auratus* for the *Control* (light grey) and *Sunscreen exposure* (dark grey) groups.** A: HSI is expressed in %; B: FI is expressed in %; C: SGR is expressed in % day<sup>-1</sup>. Values represent mean  $\pm$  standard error. Different letters above bars indicate a statistical difference ( $P < 0.05$ ).

### 3.2.3. Oxidative damage in the liver, gills and muscle of *C. auratus*

Chronic exposure to the sunscreen media solution increased cellular damage in the muscle of *C. auratus*. Indeed, MDA content in the muscle of the *Sunscreen exposure* group was statistically higher than the MDA content in the muscle of the *Control* group (*i.e.*  $0.12 \pm 0.02$   $\mu\text{mole of MDA.g}^{-1}$  of protein and  $0.08 \pm 0.01$   $\mu\text{mole of MDA.g}^{-1}$  of protein, respectively, Mann–Whitney U test,  $W = 34$ ,  $p$ -value = 0.03). In the liver and gills, no statistical difference of MDA concentrations was observed between the *Sunscreen exposure* and *Control* groups (Mann–Whitney U test,  $W = 68$  and  $p$ -value = 0.56;  $W = 88$  and  $p$ -value = 0.92, respectively).

Carbonyl content in the muscle, the liver and the gills did not differ statistically among the groups (*i.e.* T-test,  $df = 20.6$ ,  $p$ -value = 0.44; Mann–Whitney U test,  $W = 77$ ,  $p$ -value = 0.76; Mann–Whitney U test,  $W = 80.5$ ,  $p$ -value = 0.70, respectively).



**Figure 5: Oxidative damage in different organs of *C. auratus* for the Control (light grey) and Sunscreen exposure (dark grey) groups.** From the top to the bottom, the results for the muscle, the liver and the gills are presented. The left panel represents MDA concentrations, and the right panel represents the carbonyl content for each organ. Values represent mean  $\pm$  standard error. Different letters above bars indicate a statistical difference ( $P < 0.05$ ).

#### 4. Discussion

Our study had two main objectives: (1) to determine the detection abilities and spatial avoidance choice of a marine teleost species when individuals were exposed to sunscreen products containing O-UVFs and (2) to evaluate the toxicity of these contaminants by estimating the oxidative damage at the cellular level as well as by measuring the impact on the general health status, using biometric indicators measured at the individual level.

To do so, we exposed fish to environmentally realistic concentration of O-UVFs reached during summer season (i.e. few  $\mu\text{g} \cdot \text{L}^{-1}$ , Langford and Thomas 2008; Sánchez Rodríguez et al. 2015; Bratkovics et al. 2015) and to realistic duration (35 days for the 2<sup>nd</sup> experiment) of exposure. Also, we used a

realistic mode of exposure with the diurnal variation of O-UVFs observed in our experiment and *in situ* (Picot-Groz et al., 2018 and Sankoda et al., 2015).

#### 4.1. Determination of the detection abilities and spatial avoidance choice in *C. auratus* when exposed to sunscreen products

In our first experiment, we did not find any avoidance behaviour of the sunscreen products by *C. auratus* (Figure 3) when exposed to a total average O-UVFs concentration of  $3.65 \mu\text{g.L}^{-1}$ . To our knowledge, few studies have investigated the detection abilities and avoidance response of organisms when exposed to sunscreen products. Among them, no avoidance behaviour was recorded in either the flatworm *Convolutriloba macropyga* (McCoshum et al., 2016 ) or the fish *Sparus aurata* (Díaz-Gil et al., 2017) when exposed to sunscreen ( $0.13 \pm 0.07 \text{ g L}^{-1}$  and 1000 ppm, respectively). The results of these studies are in accordance with our observation in the teleost *C. auratus*. Conversely, Araújo et al., (2020) recorded an avoidance behaviour for shrimp *Palaemon varians* but at concentrations higher than those used in our study (*i.e.* from 100 to 200  $\text{mg.L}^{-1}$  of sunscreen). In this sense, it can be hypothesised that *C. auratus* did not detect the sunscreen products because of the low (although environmentally relevant) concentrations tested. Indeed, Claireaux et al., (2018) studied the avoidance process towards aromatic hydrocarbon compounds that can be considered as structurally close to O-UVFs. These authors determined that European seabass (*Dicentrarchus labrax*) could not detect or avoid water contaminated at a level below  $3 \mu\text{g.L}^{-1}$  of PAHs.

While the hypothesis previously proposed – *i.e.* the fact that concentrations used were too low to be detected – seems to be the most likely, it is also possible that exposure to the contaminants during the experiment resulted in a reduction or a suppression of the olfactory function of *C. auratus* such as observed after 10 minutes in Fathead minnows (*Pimephales promelas*) that were exposed to low copper concentrations (Green et al., 2010); after 15 or 30 minutes, respectively, in Eurasian perch (*Perca fluviatilis*) and Crucian carp (*Carassius carassius*) that were exposed to  $\mu\text{g.L}^{-1}$  concentrations of

silver (Bilberg et al., 2011); and after 5 minutes in Rainbow trout (*Oncorhynchus mykiss*) that were exposed to oil sands process-affected water (OSPW) containing hydrocarbons (Lari and Pyle, 2017).

Another possibility is that *C. auratus* detected the sunscreen products but ignored them due to the fact that the fish do not associate them with a potential risk to their health. To this point, McCoshum et al., (2016) observed that Acoel flatworms (*Convolutriloba macropyga*) exposed to 0.26 mL.L<sup>-1</sup> of sunscreen containing homosalate, oxybenzone, octocrylene, octisalate and avobenzone did not avoid this contaminant. The authors hypothesised that organisms do not perceive sunscreen as a threat. In this line of thought, Tierney, (2016) provided an explanation for the absence of avoidance of individuals exposed to some chemicals. According to the author, the chemical products responsible for causing a behavioural response in the organisms were mainly toxic contaminants that have been present in the environment for many decades (Table 1 in Tierney, 2016). This long-term contamination could have created selective pressure, resulting in the conservation of avoidance behaviours for these historical contaminants. Conversely, more recent synthetic chemicals, such as sunscreens, would not yet have led to the selection of these avoidance behaviours in the fish population.

This absence of avoidance in teleosts when exposed to sunscreen products deserves to be further investigated to understand the causes of this phenomenon. Be that as it may, these results suggest that, *in situ*, fish would not escape a contaminated area and, consequently, would be exposed to sunscreen products with the possibility of incorporating the pollutants, which may lead to adverse physiological effects. On this basis, our second experiment was aimed at investigating this potential toxicity. Several authors have already demonstrated the potential toxicity of O-UVFs, mainly on reproduction and development, but often by exposing organisms to acute concentrations of a single molecule (Carve et al., 2021). On this basis and in the aim to simulate the seasonal *in situ* exposure of organisms to O-UVFs, our second experiment permitted us to expose *C. auratus* to environmental concentrations of a cocktail of O-UVFs and excipients for 35 days. Toxicity was then determined

through the measurement of different markers for oxidative stress and the estimation of several indices that are classically used in ecophysiology and ecotoxicology to evaluate the general health of individuals.

4.2. Determination of the general health status (condition, growth and hepatosomatic index) and oxidative stress in *C. auratus* when exposed to sunscreen products

#### 4.2.1. General health status

The FI and the SGR are two complementary and commonly used indices to assess the general welfare of the fish and are an integrative estimate of environmental conditions. Regarding the FI, it provides information on overweight of the fish. A decrease of FI would indicate that the fish loses weight and, therefore, its body condition decreases. In our study, individuals exposed for 35 days to environmental concentrations of the pollutant had a similar FI to that of unexposed individuals (Figure 4B), suggesting that there was no effect from O-UVFs exposure. Several studies are in accordance with our results. For example, Ziarrusta et al., (2018) exposed the gilt-head bream *Sparus aurata* to non-environmental concentrations of Benzophenone-3 (BP-3), a commonly used O-UVFs, and no FI alteration was recorded after such exposure. In accordance with the results of Ziarrusta et al., (2018), Almeida et al., (2019) found no change in FI in the guppy *Poecilia reticulata* when exposed to the same pollutant, *i.e.* BP-3, at environmental concentrations. Similarly, Cahova et al., (2021) found that ingestion of pellets contaminated from 6.9  $\mu\text{g}\cdot\text{kg}^{-1}$  to 395.6  $\mu\text{g}\cdot\text{kg}^{-1}$  of EHMC (an O-UVF also found in our sunscreen exposure media) for 6 weeks did not cause a change in FI in Rainbow trout (*Oncorhynchus mykiss*).

Regarding the SGR, it was also not altered by the contaminant, suggesting that O-UVFs did not influence the growth of *C. auratus*. Studies regarding the effects of O-UVFs on fish growth are scarce (see the review of Lozano et al., 2020). However, the study of Araújo et al., (2021) supports our results. Indeed, these authors did not highlight a significant impact on the growth of the flatfish *Solea senegalensis* when this fish was exposed to 0.294  $\text{mg}\cdot\text{L}^{-1}$  of a commonly used UV filter, the 4-MBC.

Regarding the HSI (liver weight/fish weight) a 33 % increase was measured in individuals exposed to sunscreen. This modulation is rather due to an increase in liver weight rather than a decrease in fish weight. These results are in contrast with those obtained by Kim et al., (2014) since these authors did not identify a change in the hepatosomatic index in Japanese medaka (*Oryzias latipes*) that had been exposed for 14 days to water contaminated with BP-3. In the same way, Yan et al., (2020) found that 50  $\mu\text{g.L}^{-1}$  of OC did not modulate the HSI in the Japanese medaka (*Oryzias latipes*) after 28 days of exposure. So, while these last authors did not find an effect at concentrations of OC of 50  $\mu\text{g.L}^{-1}$ , we found an effect for lower concentrations of O-UVFs. This could be due to the toxicological effect of other O-UVFs than OC (such as EHMC or EHS which were present in our exposure media). Another explanation could be the cocktail effects of several molecules (O-UVFs but also excipients) in the exposure media (as already suggested by Park et al., 2017; He et al., 2019; Carve et al., 2021).

Regarding our results, we can first hypothesize that this increase in HSI could be linked to the initiation of the detoxification processes. In order to answer this hypothesis, it would be interesting to measure biotransformation enzymes like EROD (ethoxyresorufin-O-dealkylase). Sinaei and Mashinchian, (2014) measured a positive correlation between HSI and the total concentration of low molecular weight PAHs, that are partially resulting from detoxification processes in the mudskipper species (*Boleophthalmus dussumieri*).

Also, we can hypothesize that this higher HSI could be due to liver pathologies. In this sense, several authors showed that exposure to various pollutants induced histopathological alterations (e.g. hepatocyte hypertrophy, vacuolisation, congestion, fibrosis) that were associated with an increase in HSI in the freshwater cyprinid *Alburnus mossulensis* (Dane and Sisman, 2020), the Guinean tilapia *Tilapia guineensis* and the African catfish *Clarias gariepinus* (Agbohessi et al., 2015). Other authors suggest that this increase of HSI after pollutant exposure could be linked with lipid accumulation in the liver (Authman, 2011). Consequently, regarding the potential effects of the O-

UVFs on the liver of fish, further investigations are needed in order to understand the toxicological mechanisms of action as well as to evaluate the loss of functional integrity of this organ.

While the results of our study exposed the effects at the individual level, it is generally admitted that biological markers assessed at a lower scale are more sensitive and more precocious (Milinkovitch et al., 2019) and, thereby, can be used for a more accurate assessment of the toxicological impact. On this basis, the effects of sunscreen products were also analysed at the subcellular level with the evaluation of oxidative damage.

#### 4.2.2. Oxidative damage

In this study, we measured two biomarkers for oxidative damage in the liver, the gills and the muscle of *C. auratus*: the cellular content of MDA, which is an estimate of lipid peroxidation (Tsikas, 2017), and the level of protein carbonylation, which partly reveals free radical attack of cellular proteins (Parvez and Raisuddin, 2005). An increase in MDA level and/or protein carbonylation is usually measured during exposure to pollutants (*e.g.* Almroth et al., 2005; Solomando et al., 2020; Santillán Deíu et al., 2021; Farag et al., 2022) and reveals the oxidative damage due to an imbalance between pro-oxidants (ROS) and antioxidants (such as enzymatic or non-enzymatic ones) in favour of the pro-oxidants (Qu et al., 2014).

In our study, we did not show that contaminant induced oxidative damage in the liver of *C. auratus*, *i.e.* no increase in the level of MDA nor in the carbonyl content (Figure 5), which could be due to the intervention of antioxidant defences. Indeed, an increase in antioxidant systems following contamination with O-UVFs has been shown by some authors. For example, Cahova et al., (2021) recorded an increase in the activity of glutathione peroxidase (GPx) and, therefore, did not record an increase in thiobarbituric acid reactive substances TBARS (markers of lipid peroxidation) in the liver of rainbow trout (*Oncorhynchus mykiss*) when exposed for 6-weeks to pellets contaminated with 6.9  $\mu\text{G.kg}^{-1}$  of EHMC. In the same way, Ma et al., (2017) measured an increase in CAT in the liver of crucian carp (*Carassius auratus*) that were exposed for 28 days to a mixture of BMD-BM and OD-



PABA of a magnitude of  $20 \mu\text{g}\cdot\text{L}^{-1}$ . Finally, these results suggest that the increase in HSI we measured was not due to oxidative stress.

Regarding the gills, numerous studies have highlighted that they are a target organ for various pollutants (Barboza et al., 2018; Ma'rifah et al., 2019; Sachi et al., 2021). However, very few studies have considered the oxidative stress due to O-UVFs on this organ. In our study, we observed no alteration of the levels of MDA and carbonyl content (Figure 5), which could suggest an absence of ROS production or an increase of antioxidant defences in order to prevent the occurrence of oxidative damage.

For the muscles, no change in carbonyl content (Figure 5) was measured in *C. auratus* when exposed to the sunscreen exposure media. However, regarding MDA, we measured a significant increase for fish of the *sunscreen exposure* group (Figure 5). This phenomenon could be due to an excess of ROS production but, alternatively, to a decrease of the antioxidant capacity following the contamination. In this line, the study by Nataraj et al., (2020) suggests that a decrease in SOD would be at the origin of lipid peroxidation and degeneration of muscle cells in zebrafish embryos exposed for 96h to a concentration of  $3.5 \mu\text{g}\cdot\text{mL}^{-1}$  of 4-MBA, a photoproduct of O-UVFs. A more likely explanation could be that oxidative damage was only observed in the muscle – but not in the liver or gills of *C. auratus* – due to a differential bioaccumulation of O-UVFs between these organs. Indeed, Molins-Delgado et al., (2018) noted that two of the four O-UVFs present in the sunscreen exposure media used in our study (*i.e.* OC and EHMC) tend to accumulate preferentially in the muscle rather than in the liver and gills of the lebranche mullet (*Mugil liza*) which was sampled in a highly urbanised Bay of Rio de Janeiro (Brazil). Be that as it may, regarding these results, our study first suggests that the measurement of MDA in the muscle is of major interest in view of its potential sensitivity to O-UVFs. Moreover, the increase in MDA level does not appear to have impacted the SGR nor the FI of fish exposed to the sunscreen. It is possible that there was no repercussion of oxidative stress at the individual level or that we did not measure the biological parameters highlighting these

repercussions. So that, it would be of interest to accentuate studies focusing on the impact of O-UVFs upon the functional integrity of muscle.

## 5. Conclusion

The first part of our study showed that *C. auratus* did not avoid the sunscreen contaminant. Although the underlying physiological mechanisms involved in this behaviour deserve further investigation, it could be stated that this lack of avoidance would lead, *in situ*, to fish contamination and, consequently, to potential adverse physiological effects. Thus, the second part of our study – which simulated a realistic summer contamination in terms of duration, diurnal variation and environmental concentrations of O-UVFs – highlighted this toxicological impact. The multi-scale approach allowed us to use both ecologically relevant and ecotoxicologically sensitive biomarkers (such as growth and oxidative stress indicators, respectively). At the individual level, it appeared that contaminants had no effect on the growth or on the condition index of *C. auratus*, suggesting no negative consequence of these pollutants upon higher levels of biological organisation (such as population). However, the hepatosomatic index increased after 35 days of exposure. This increase of the HSI could be associated with histopathological alterations. Thus, it would be relevant to further analyse the effects of these sunscreen products on the functional integrity of the liver.

At the cellular level, concerning oxidative stress, we did not reveal oxidative damage in either the liver or the gills. We could, therefore, suggest that, in the liver and the gills, ROS production was too low to induce oxidative stress or that antioxidant defences eliminated the ROS. Measuring these defences could validate this hypothesis. For muscle, an increase in the level of MDA was measured in exposed individuals, which may be linked to a failure of antioxidant defences and/or an excess of ROS production. The fact that we measured oxidative damage in muscle, but not in gills or liver, is probably due to the preferential accumulation of O-UVFs in this organ. Regarding these results, muscle appears to be an organ to further consider in O-UVFs ecotoxicological studies. For instance, it would be relevant to establish whether exposure to these contaminants had an impact on the

cellular component of muscle tissue and how such alterations could have repercussions on individual performance such as swimming, mating or foraging.

Finally, it should be stated that the effects observed in our study could also be due to other compounds than the O-UVFs, such as the excipients of the sunscreen product. Also, excipients could interact with O-UVFs and enhance the toxicity of the latter. More investigations would be necessary to understand the effect of these excipients on marine organisms.

Moreover, it should be noted that our study did not take into account the superimposition of several stressors. Indeed, other stressors present during the summer period, such as extreme temperature or oxygen depletion events, may interact with O-UVFs molecules and change their effects on organisms. Furthermore, other cosmetic products may be released at sea during sunbathing activities, suggesting potential synergetic effects of these products with O-UVFs. Such a multi-stress approach would allow us to better estimate the response of organisms facing exposomes from the coastal areas under anthropogenic activities.

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