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Effect of 10 UV filters on the brine shrimp *Artemia salina* and the marine microalga *Tetraselmis* sp.

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Abstract: The presence of pharmaceutical and personal care products' (PPCPs) residues in the aquatic environment is an emerging issue due to their uncontrolled release, through grey water, and accumulation in the environment that may affect living organisms, ecosystems and the public's health. The aim of this study is to assess the toxicity of benzophenone-3 (BP-3), bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT), butyl methoxydibenzoylmethane (BM), methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT), 2-Ethylhexyl salicylate (ES), diethylaminohydroxybenzoyl hexyl benzoate (DHHB), diethylhexyl butamido triazone (DBT), ethylhexyl triazone (ET), homosalate (HS), and octocrylene (OC) on marine organisms from two major trophic levels including autotrophs (*Tetraselmis* sp.) and heterotrophs (*Artemia salina*). In general, results show that both HS and OC are the most toxic UV filters for our tested species, followed by a significant effect of BM on *Artemia salina* due to BM but only at high concentrations (1 mg/L) and then an effect of ES, BP3 and DHHB on the metabolic activity of the microalgae at 100 µg/L. BEMT, DBT, ET, MBBT had no effect on the tested organisms, even at high concentrations (2mg/L). OC toxicity represent a risk for those species since concentrations used in this study are 15-90 times greater compared to those reported in occurrence studies for aquatic environments. Reported for the first time the HS toxicity on a microalgae species at concentrations complementing those found in aquatic environments. These preliminary results could represent a risk in the future if concentrations of OC and HS continue to increase.

Keywords: UV-filters**;** Toxicity tests**;** Marine microalgae**;** *Artemia salina***;** Marine environment

1. Introduction

In recent decades, the sunscreen production has continuously increased with the rise of awareness to protect the skin against damaging sunlight exposure and to reduce the risk of skin cancer [1,2]. Sunscreen products contain many chemical compounds and the active ingredient being UltraViolet (UV) filters, the aim of which is to absorb or reflect UVA and/or UVB radiations ranging from 280 to 400 nm [3].

In 2016, 60 different UV filters were reported on the market and these compounds are subject to different regulations around the world [4]. UV filters are regularly detected in various aquatic environmental compartments including lakes, rivers, surface marine waters and sediments [3,5-7]. Some UV filters have been investigated more than others in occurrence studies, such as : benzophenone-3 (BP3), ethylhexyl methoxycinnamate (EMC), octocrylene (OC) and 4-methylbenzylidene camphor (4-MBC). In marine coastal waters most of the filters occur at concentrations in the range of 0.1-10µg/L [5-7]. Concentrations as high as 1.4 mg/L was reported for BP3 in the U.S. Virgin Islands' coastal waters [8]. These chemicals can enter in the marine environment in two ways, either indirectly from from the effluent of wastewater treatment plants or

directly from swimming or recreational activities [9]. Furthermore, their lipophilic nature results in low water solubility, high stability and tendency to bioaccumulate [10,11].

To investigate the impact of these UV filters on the environment, ecotoxicological studies have examined various trophic levels, from microalgae, coral to fish. Several studies have demonstrated that some of these compounds can disrupt survival [12-14]. behavior [15,16], growth [14,17,18], development [19,20], metabolism [21-23], gene expression [24,25] and reproduction [15,26,27] in various species. It should be noted that the majority of toxicological studies on organic UV filters were conducted on BP3, EMC and 4-MBC [7]. BP3 and EMC have been banned in Hawaii and Key West, Florida The Republic of Palau and U.S Virgin Islands have also banned these two UV-filters, as well as OC The adoption and implementation of the European legislation on the registration, evaluation, authorization and restriction of chemicals (REACH) requires several additional ecotoxicity data and promoting the use of invertebrates as models for toxicity assays [28].

The aim of this present study was to evaluate the toxicity of ten common UV filters : benzophenone-3 (BP-3), bis-Ethylhexyloxyphenol methoxyphenyl triazine (BEMT) butyl methoxydibenzoylmethane (BM), diethylaminohydroxybenzoyl hexylbenzoate (DHHB), diethylhexyl butamido triazone (DBT), ethylhexyl salicylate (ES), ethylhexyl triazone (ET), homosalate (HS), methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) and octocrylene (OC) on two model organisms commonly used in ecotoxicity assays [22,29-31] : the green algae *Tetraselmis* sp., a primary producer commonly used for chronic algal toxicity [22] and the brine shrimps *Artemia* spp. (here *A. salina*) readily available worldwide and easy to breed. Any alterations in these populations may result in chain reaction effects on organisms at higher trophic levels [22,31]. This study is a preliminary study before investigating a large diversity of species.

2. Materials and methods

2.1. Test substances and experimental solutions

The UV filters benzophenone-3 (BP-3), bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT), butyl methoxydibenzoylmethane (BM), and methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). 2-Ethylhexyl salicylate (ES), diethylaminohydroxybenzoyl hexyl benzoate (DHHB), diethylhexyl butamido triazone (DBT), ethylhexyl triazone (ET), homosalate (HS), and octocrylene (OC) were provided by Pierre Fabre Laboratories.

Before each toxicity test, and due to the low water solubility of the compounds, stock solutions at 1 mg/ml were prepared by dissolving each UV filters in dimethyl sulfoxide (DMSO, Sigma-Aldrich, purity >99%). These solutions were diluted in order to add the same amount of DMSO to all samples and to obtain exposure concentrations ranging from 20 ng/L to 2 mg/L for *A. salina*, and 10 µg/L to 1 mg/L for *Tetraselmis* sp.. The lower concentrations tested were roughly those reported in marine ecosystems (0.1-10 µg/L). For *Tetraselmis* sp. three concentrations were selected to investigate on a possible dose-response effect. The lowest tested concentration was the highest concentration reported in the environment for most filters. This assay on *Tetraselmis* sp. is considered as a preliminary study before investigating the response of phytoplankton on a wide phylogenetic diversity of species. DMSO concentration in the experiments was always 2.5 % (v/v). A DMSO control (2.5 % v/v) and a blank control were also included. The blank control was artificial seawater (ASW) for *A. salina* and growth medium for *Tetraselmis* sp..

2.2. Artemia salina mortality test

A. salina cysts were purchased from AquarHéak Aquaculture (Ars-en-Ré, France) and stored at 4 °C. Dried cysts were hatched in a constantly aerated transparent «V» hatching incubator filled with 500 mL of artificial seawater (ASW) at a salinity of 37 g/L, prepared with Instant Ocean salt (Aquarium Systems, Sarrebourg, France). Incubation was carried out for 48h, at 25 °C under continuous light the first 24 hours. A 12:12 h light regime was then applied until the nauplii reached

92 the instar II-III stage. Ten nauplii were transferred into 5 ml glass tubes filled with ASW (2 mL)
93 supplemented with DMSO and UV filters. The tubes were incubated at 25 °C under a 12:12 h light
94 regime. The experiments were performed in sextuplicate. During the exposure period, there was no
95 aeration and the nauplii were not fed. The mortality rate was estimated after 48 hrs by counting the
96 dead nauplii under binocular. Organisms with no swimming activity or movement of appendices for
97 10s even after mechanical stimulation with a Pasteur pipette were counted as dead. The tests were
98 considered valid if the control's average mortality rate was < 20%.

99 *2.3. Tetraselmis sp. toxicity test*

100 2.3.1. Experimental procedure

101 *Tetraselmis* sp. (RCC500) was purchased from the Roscoff culture collection and was grown in
102 filtered (pore size: 0.22 µm) and autoclaved seawater enriched with a 50-fold diluted f/2 medium
103 (Sigma–Aldrich). The culture was maintained under controlled conditions at 18 °C (± 1 °C) with a
104 photon flux of 70 µmol photons.m^{-2}.s^{-1} under a dark:light cycle of 12:12h. Toxicity tests were
105 conducted in 150 mL Erlenmeyer flasks containing 50 mL of culture. Algae cells in exponential
106 growth phase were used as inoculum with an initial cell density of 5.10^4 cells/mL. Three replicates
107 per UV filter concentration were performed. After 7 days of exposure, different morphological and
108 physiological cells properties were monitored *via* flow cytometry (FCM). Analyzed parameters were
109 granularity, relative cell volume, chlorophyll a fluorescence, esterase activity, and growth. The
110 control experiment was a *Tetraselmis* sp. culture supplemented with DMSO (2.5%).

111 2.3.2. Flow cytometry (FCM) analyses

112 Aliquots were collected after seven days of exposure to be analyzed in a FACSCanto II flow
113 cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA) equipped with an air-cooled argon
114 laser (488 nm, 15 mW). To characterize the microalgae population, and to exclude non-algal particles,
115 the forward scatter (FSC, an estimation of cell size) and side scatter (SSC, an estimation of granularity)
116 dot-plots were established before each measurement. The flow rate of the cytometer was set to low
117 with an acquisition time of 1 min.
118 The data recorded by FCM were measured directly (autofluorescence, granularity, size) and
119 indirectly by the use of fluorochromes (esterase activity). Cellular density was determined using
120 Becton Dickinson Trucount™ 10 µm beads for calibration, as already described [32]. Growth rate (µ),
121 expressed as day^{-1} were calculated using the following equation: $\mu = (\ln(N_t)-\ln(N_0)) / (t-t_0)$, where N_t
122 is the cell density at time t and N_0 is initial cell density. Chlorophyll a autofluorescence was measured
123 and detected in the FL3 channel. Relative cell volume (size) and granularity were directly estimated
124 with the forward light scatter (FSC channel) and with the side scatter channel (SSC), respectively. The
125 metabolic activity was determined based on the esterase activity. Cellular esterase activity
126 measurement is a method frequently used to determine the metabolic activity of cells [33]. The most
127 common substrates used are acetomethyl esters (calcein-AM and (BCECF-AM) and fluorescein
128 diacetate (FDA) and its various substituted derivatives. Once inside the cell, the substrate is
129 converted by intracellular esterases into calcein or fluorescein that has a net negative charge at neutral
130 pH. Thus, the fluorescent molecules are maintained inside the cell by the intact membrane potential
131 and therefore, the concentration of fluorescein (or derivatives) trapped in the cell increases with time.
132 In this study cells were stained with the fluorochrome Chemchrom V6 (10-fold diluted in ChemSol
133 B26 buffer– Biomérieux, France) at 1 % final concentration, and incubated for 15 min at room
134 temperature in the dark before analysis using the FL1 channel. All cytometry data were analyzed

using BD FACSDiva (Becton Dickinson). Results were expressed as percentage of variation relative to the control (100 %).

2.4. Statistical analysis

Results are reported as mean and standard deviation (SD), calculated from the 3 or 6 replicates. For both tests and all the parameters measured, differences between controls and nominal concentrations of UV filter were analyzed using R software, by one-way analysis of variance (ANOVA) followed by post-hoc Tukey HSD tests for pairwise comparisons. In all cases, significance was accepted when $p < 0.05$. Half maximal lethal concentration (LC_{50}) is the concentration that induces 50 % mortality in *A. salina* (for BM, HS, and OC) or *Tetraselmis* sp. (for HS only). Half maximal effective concentration (EC_{50}) refers to the concentration that induces a response halfway between the baseline and maximum. EC_{10} corresponds to the concentration that affect 10 % of the population. LC_{50}/EC_{50}-values were estimated with a log(agonist) vs. response - Variable slope (four parameters) regression model in GraphPad Prism 5.

3. Results and discussion

3.1. Effects on Artemia salina mortality

The toxicity of several organic UV filters on the marine crustacean *Artemia salina* (Nauplii Instar II/III) was determined after a 48h exposure by counting dead larvae (Fig. 1). At the highest concentration tested (2 mg/L), HS, BM, and OC demonstrated a significant effect on Nauplii survival ($p < 0.05$) with mortality values reaching 54 ± 16%, 64 ± 19% and 88 ± 16%, respectively. At lower concentrations of these filters no significant effect was detected. For BP3, BEMT, MBBT, ES, DHHB, DBT and ET no toxicity was observed, even at the highest concentration.

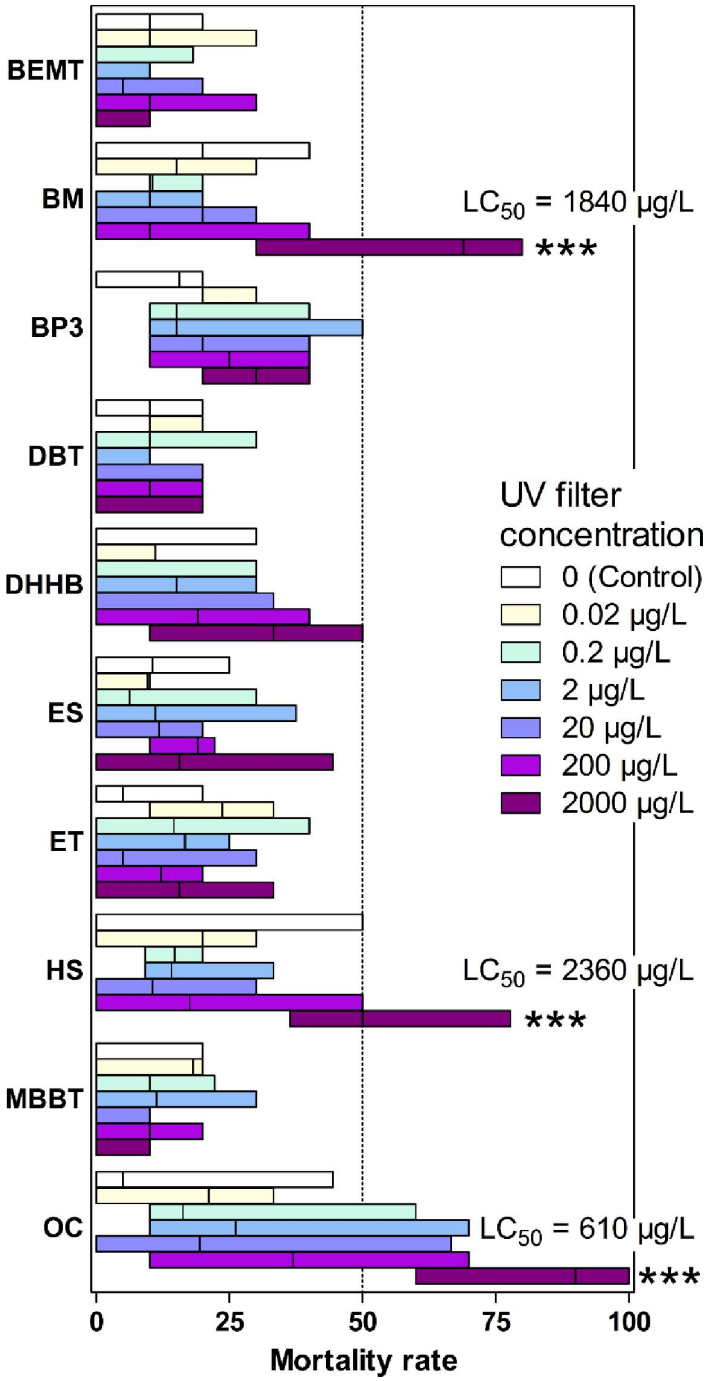

Figure 1. Mortality rate of *A. salina* exposed to the 10 UV filters at 6 concentrations. Boxes delineate the minimal and maximal values, and the vertical line is the median of six replicates. Significance levels relative to control determined by ANOVA followed by the Tukey's multiple comparison test: *** $p < 0.001$. Results were not significant unless otherwise stated. For BM, HS and OC, the LC_{50} is reported on the figure.

Our results indicate that among the different UV filters tested in this study, OC was the most toxic molecule showing the lowest LC_{50} concentration (0.6 mg/L), followed by BM and HS (1.8 mg/L and 2.4 mg/L, respectively). Environmental HS and BM concentrations reported so far are at least 500 times lower than LC_{50}, with values lower than 3 µg/L [3,5,34,35]. OC concentrations in coastal waters

are higher and have been detected up to 9 μg/L [5,35,36]. This is the first report showing OC toxicity on *Artemia salina*. We also observed a concentration-dependent increase in mortality of *Artemia* with respect to the control. This is congruent with the toxicity observed, at lower concentration, on coral (50 μg/L) [23], urchin, mussel and algae (40-80 μg/L) [19]. OC also affects the developmental process in zebrafish [37]. Here the LC_{50} on *A. salina* is approximately 90 times higher than the highest OC concentrations in marine waters reported so far. It should be mentioned as well that OC concentrations in the 50-100 μg/kg range have been frequently reported in sediments [38,39]. OC is a pseudo-persistent pollutant; its contamination of the environment is refreshed daily. As such, it may indeed affect benthic crustacean.

3.2. *Effects on Tetraselmis* sp.

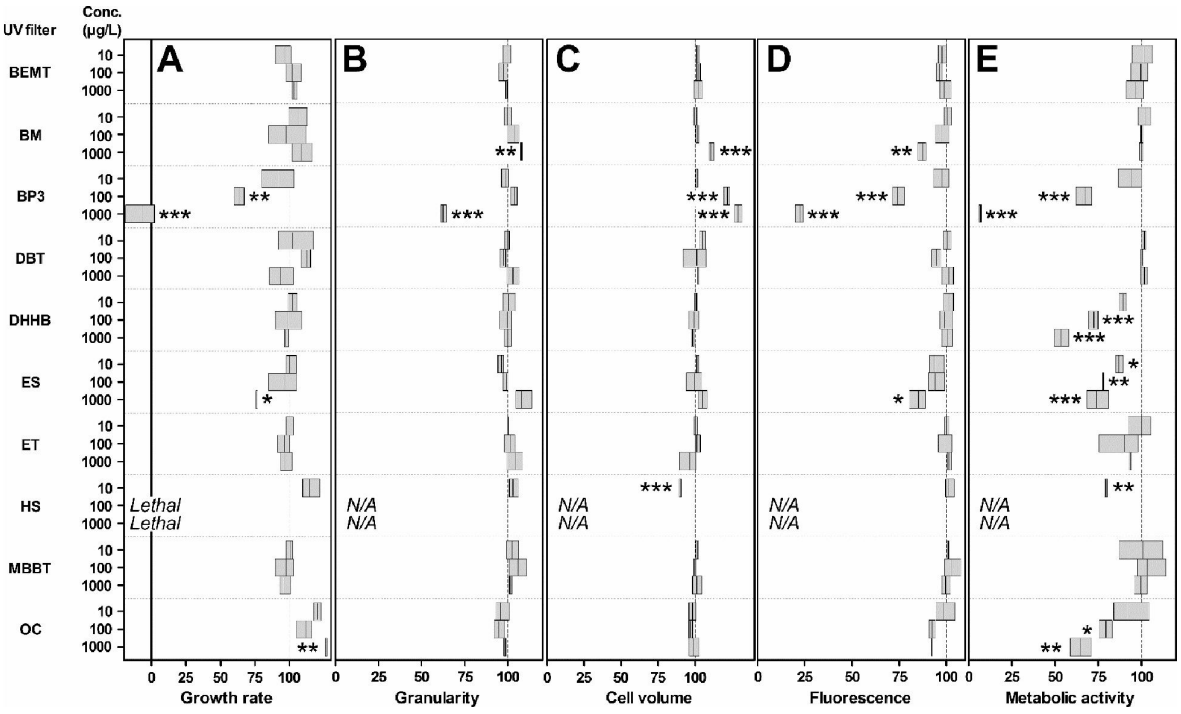

Figure 2. Relative A) growth rate, B) granularity, C) cell volume, D) fluorescence and E) metabolic activity of exposed *Tetraselmis* compared to control, set to 100 %. The boxes delineate the minimal and maximal values. The vertical line in the boxes is at mean. Significance levels relative to negative control determined by ANOVA followed by the Tukey's multiple comparison test: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Results were not significant unless otherwise stated. N/A: not applicable, the data could not be obtained due to extensive cell death.

3.2.1. Growth rate and EC_{50} values

After 7 days of exposure, HS, BP3, and ES induced a significant decrease of algae growth (Fig. 2A). The growth rate of algae exposed to ES at 1 mg/L decreased by 24 % compared to control ($p < 0.05$). Data for the presence of ES in the environment are scarce. ES concentrations in coastal seawater have been reported in the range 1-1030 ng/L [5,40]. At these concentrations our results suggest that ES may have no impact on *Tetraselmis* sp.; it is crucial though to determine its toxicity on a wide range of phytoplankton species before concluding. For BP-3 we observed a concentration dependent decrease in growth, which was statistically significant at 100 μg/L ($p < 0.01$) and 1 mg/L ($p < 0.001$). At 1 mg/L, the growth rate was negative, which translates in a decreased cell concentration compared to t_0. The 7-days EC_{50} value for BP3 was 143 μg/L. BP3 EC_{50} values on several microalgae species have been reported previously to be roughly in the 100 μg/L to 20 mg/L range [17,21,41,42]. Seawater BP3

concentrations in the μg/L range have been frequently reported in the literature [5,10]. Extremely high values of 1.4 and 0.6 mg/L have been recorded in the U.S. Virgin Islands [8]. At such high concentrations, BP3 is highly toxic for *Tetraselmis* sp.. Finally, the most important decline was with the UV filter HS. No algal cells were detected in the presence of HS at 100 μg/L and 1 mg/L. LC_{50} with HS was estimated at 74 μg/L, while it has been shown that HS concentration in aquatic environments can reach ~3 μg/L [5,35]. Further experiments should be conducted on a wider diversity of phytoplankton species and at lower concentrations to better interpret the toxicity of HS and its potential impact of phytoplankton communities. If toxic to Symbiodiniaceae, HS may also contribute to coral bleaching. OC induced a slight but significant increase for the growth rate at 1 mg/L. The increased growth rate may be due to a hormesis effect. Nonetheless, the decreased metabolic activity at day 7 is a significant sign of toxicity detectable at 100 μg/L and above. Similar differences in the response of different physiological parameters were already reported [21] for the toxicity of BP3 in the microalgae *Chlamydomonas reinhardtii*. The growth rate of *Tetraselmis* was not affected by BEMT, BM, DBT, DHHB, ET and MBBT, even at 1 mg/L. To the best of our knowledge, there are no data on the toxicity of these filters on phytoplankton species. As mentioned above, these results should be considered as preliminary data and more assays should be performed on a wide diversity of species. Co-occurrence of these filters should be investigated with many sunscreen brands containing different UV filters and often a few UV filters in the one product.

3.2.2. Impact on cell morphology

Three of the UV filters induced cell morphological alterations (Fig. 2B/C). Cells cultured in the presence of BM have experienced a significant increase in cell volume and granularity at 1 mg/L ($p < 0.05$). This concentration is 1,000 to 10,000 times higher than the few concentrations reported in the field [3,5,34]. According to environmental concentrations of other UV filters, one can assume that the effective concentration of 1 mg/L is probably higher than any BM environmental concentration, but this remains to be confirmed. BP3 caused a dose dependent increase of relative cell volume at 100 μg/L and above, reaching up to 129 % of control cell volume at 1 mg/L ($p < 0.001$). Meanwhile, this UV filter induced a significant 38 % granularity decrease at 1 mg/L ($p < 0.001$). With reference to the environmental concentrations of BP3 (see above), this filter should exert a significant impact on phytoplankton communities. These results are congruent with what was recently reported [42] on *Arthrospira* sp. With HS, cell volume and granularity could not be measured at 100 and 1000 μg/L. However, a significant cell volume decrease was observed at 10 μg/L of HS (– 10.2 %, $p < 0.001$). Here, the EC_{10} value is slightly lower than 10 μg/L, *i.e.*, within the same order of magnitude than the highest water column concentration reported so far [5]. Again, it is expected that HS should affect microalgae communities in bathing areas. No significant effect was observed for BEMT, DBT, DHHB, ES, ET, MBBT and OC. As mentioned above, there are no data on the toxicity of these filters on the morphology of phytoplanktonic cells but further investigations on other species are needed to conclude on their toxicity on phytoplankton.

3.2.3. Impact on autofluorescence

The results of FCM analysis revealed that several UV filters significantly reduced chlorophyll a (Chl a) cell fluorescence (Fig. 2D). The decrease was significant with BM (–13 %, $p < 0.01$) and ES (– 15 %, $p < 0.05$) at 1 mg/L. A strong dose-dependent autofluorescence inhibition was observed upon exposure to BP3 at concentrations of 100 μg/L ($p < 0.001$) and above. Inhibition reached 78 % at the highest dose. Again, autofluorescence could not be measured in cells treated with HS at 100 and 1000 μg/L due to the cell degradation at these concentrations. No significant effect was observed for BEMT, DBT, DHHB, ET, MBBT and OC. Again, this is the first report that shows results on the effect of BEMT, MBBT, DHHB, ET and DBT on phytoplankton. These results further demonstrate that it is important to follow different parameters since OC for instance has no impact on the fluorescence but has an impact on the growth rate and metabolic activity.

243 3.2.4. Impact on cell metabolic activity

244 Metabolic activity was determined by estimating the relative esterase activity in exposed cells
245 compared to control. It was measured by CV6 staining and highlighted significant decreases in
246 metabolic activities with half of the tested UV filters (Fig 2E). Algae exposed to ES and HS
247 experienced a decreased esterase activity at 10 µg/L. The effect of BP3, DHHB and OC was significant
248 at 100 µg/L and above. A similar decreased esterase activity was reported for *C. reinhardtii* exposed
249 to BP3, although at concentrations in the mg/L range [21,43]. For DHHB, the effect was only observed
250 for the microalgae and for the esterase activity but not for other parameters. Therefore, the
251 environmental risk cannot be estimated since natural concentrations have never been reported for
252 this UV filter. No significant effect was observed for BEMT, MBBT, DBT, ET and BM.
253 The toxicity of the different UV filters is not the same between phytoplankton and zooplankton
254 and probably varies between species within the same group. This means that it is important to study
255 the response of several species within a given group. For *Tetraselmis* sp. we see that the toxicity vary
256 according to the examined parameters. Therefore, it is important to measure several parameters. For
257 example, this is clear when we look at the results of the fluorescence parameter. This could imply
258 that when the impact of UV filters on corals is analyzed based on the simple fluorescence parameter
259 of the zooxanthellae (symbionts), this approach is too simplistic because zooxanthellae can perhaps
260 react on other parameters than fluorescence.

261 **4. Conclusion**

262 The present work demonstrates that several UV filters exert toxicity on *A. salina* and *Tetraselmis*
263 sp. HS was the most toxic UV filter for the microalgae. EC_{50} was 74 µg/L and significant adverse
264 effects were recorded at the lowest concentration tested (10 µg/L). HS was also toxic for *A. salina*,
265 although at much higher concentrations (LC_{50} 2.4 mg/L). Since HS concentrations up to 3 µg/L have
266 been reported in aquatic environment, HS may represent a potential risk for marine phytoplankton
267 communities. Further research is needed to investigate the HS toxicity with a larger diversity of
268 phytoplankton species.
269 OC was toxic on both models with a dose-dependent effect on the microalgae. OC significantly
270 altered *Tetraselmis* sp. metabolic activity at 100 µg/L. On *A. salina*, LC_{50} was 610 µg/L. Overall, OC
271 toxicity was observed at 100 µg/L with these models. The toxicity of OC occurred at concentrations
272 90 (*A. salina*) and 15 (*Tetraselmis* sp.) times higher than the highest environmental concentrations
273 reported so far. These results highlight a potential toxicity of OC on marine organisms as input
274 concentrations continue to increase. BM was toxic towards the brine shrimp at high concentrations
275 with a LC_{50} of 1.84 mg/L and had little effect on the microalgae. BM toxicity was observed at 1 mg/L
276 for the algae. Such high concentrations has never been reported in the occurrence studies, although
277 the presence of this filter should be monitored in a large range of ecosystems to better interpret its
278 potential toxicity. ES, BP3 and DHHB had a significant impact on the microalgae metabolic activity
279 at concentrations between 10 and 100 µg/L but had little effect on *A. salina*
280 Overall, this research supports the need of establishing environmental quality standards for UV-
281 filters based on toxicity testing with key marine organisms, as well as identifying and reducing
282 potential toxic UV filters from entering the environment. There are still many UV filters for which
283 environmental concentrations are missing therefore the potential to estimate the environmental risks
284 are still lacking for coastal ecosytems. Based on toxicity results, it is urgent to design new
285 environment friendly UV filters.

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