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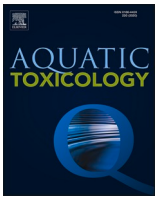

Cytotoxicity of BP-3 and BP-4: Blockage of extrusion pumps, oxidative damage and programmed cell death on *Chlamydomonas reinhardtii*

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ABSTRACT

The health concern associated with the dangers related to exposure to UV radiation has led to an increase in the use of sunscreens containing UV-filters that can reach aquatic environments and possibly affect ecosystems. Benzophenone-3 (BP-3) and benzophenone-4 (BP-4) are two of the most used UV-filters. In the present work, the microalga *Chlamydomonas reinhardtii* was exposed to several concentrations of both chemicals. To evaluate their potential cytotoxicity on microalgal cells, different parameters were analysed including fast response biomarkers (increase in intracellular free Ca^{2+}) as well as biomarkers related with the presence of oxidative stress (lipid peroxidation), energy metabolism (photosynthetic yield and cytoplasmic lipid accumulations), cell division (proliferation and F-actin content), programmed cell death (PCD) (caspase activation and DNA fragmentation) and possible mechanisms of resistance to xenobiotics (operation of extrusion pumps and presence of autophagic vacuoles). Results showed an increment of the percentage of cells with cytosolic free Ca^{2+} that could act as a secondary messenger in response to the stress. A decrease in photosynthetic yield and an increase in cytoplasmic lipid accumulations and lipid peroxidation levels were also detected. In addition, a decrease in cell proliferation was observed, linked to a decrease in the percentage of cells with F-actin. The increase observed in the microalgal population with caspase activity, together with the DNA fragmentation and the alterations in the cytoskeleton, suggested the induction of processes linked to PCD. Moreover, a blockage of extrusion pumps, which could be related to the toxicity mechanism of these compounds, and an increase in autophagic vacuoles, as an attempt to repair the damage caused by benzophenones, were detected. Overall, these biomarkers indicate that both UV-filters can be a serious threat to non-target photosynthetic microorganisms in aquatic environments, although BP-3 affected *C. reinhardtii* more markedly.

1. Introduction

The concern associated with the damage related to the exposure to UV radiation in human health has caused an increase in the use of sunscreens, which contain UV-filters that act by blocking the passage of the UV rays (Balmer et al., 2005; Jurado et al., 2014). Benzophenones (BPs) are organic compounds that absorb the UV radiation and stabilize it due to their aromatic structures (Jeon et al., 2006; Kim and Choi, 2014; Morrison et al., 2017; Suzuki et al., 2005). Chemically, BPs have the same skeleton, two benzene rings linked by a carbonyl group, but they differ in the chemical substituents of their rings (Huang et al., 2018). Nowadays, BPs are included in many personal care products (PCPs) such as sunscreens, lotions, shampoos and even in agricultural chemicals, plastic bags, and clothes, among others. Due to their extensive use, this type of UV-filters is detected in wastewaters, rivers, lakes, and oceans (Balmer et al., 2005; Ekowati et al., 2016; Sánchez Rodríguez et al., 2015), and depending on the location and the season, BPs can reach levels of mg L^{-1} (Careghini et al., 2015; Kim and Choi, 2014; Tsui et al., 2014). Among BPs, benzophenone-3 (BP-3) and benzophenone-4 (BP-4) are two of the most used (De *et al.,* 2013; Li *et al.,* 2016) and detected in aquatic environments (Negreira *et al.,* 2009). In a recent worldwide study about the occurrence of 25 organic UV-filters allowed in EU, BP-3 was reported to be the most frequently detected compound in different environmental aquatic matrices (Cadena-Aizaga et al., 2020). Regarding BP-4, this compound is the most concentrated UV-filter in wastewaters samples on the NW of Spain (Rodil et al., 2012).

BPs are listed as emerging contaminants in European aquatic environments, (NORMAN, 2022) and the toxicity of these chemicals are subject to evaluation by environmental monitoring programs according to the REACH (Regulation EC No. 1907/2006). Due to the low

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hydrophobicity and degradation rate of UV-filters (Du et al., 2017), these compounds tend to accumulate in the environment (Tsui et al., 2014; Wu et al., 2018) and in living organisms (Alonso et al., 2015; Tsui et al., 2017; Vidal-Liñán et al., 2018). Moreover, their potential toxicity has been studied on organisms of different trophic levels, such as cyanobacteria, microalgae, mussels, crustaceans, sea urchins, and fishes (Du et al., 2017; Mao et al., 2017; Paredes et al., 2014; Petersen et al., 2014; Yan et al., 2022).

Concerning the potential deleterious effects of these BPs on aquatic primary producers, microalgae have been recently found to be sensitive to the toxic impact of BP-3 and BP-4 since both BPs inhibit the population growth of several freshwater and marine microalgal species (Du et al., 2017; Huang et al., 2018; Mao et al., 2017; Petersen et al., 2014; Seoane et al., 2017; Sieratowicz et al., 2011). In a previous study, Esperanza et al. (2019) reported that BP-3 and BP-4 caused acute effects on several physiological and biochemical processes on the freshwater photosynthetic microorganism *Chlamydomonas reinhardtii*. Obtained results showed that sublethal exposure to both BPs induces a decrease in esterase activity and intracellular pH, an increase in reactive oxygen species (ROS) production on microalgal cells as well as a severe alteration of cellular membranes, including chloroplast, cytoplasmic, and mitochondrial ones. Interestingly, it is well-known that mitochondrial dysfunction plays a leading role in mediating programmed cell death (PCD) processes via ROS overproduction, cytosolic Ca^{+2} signalling, and proteolytic enzymes (caspases) and endonucleases activation in many types of cells (Farkhondeh et al., 2020). In recent years, PCD events have been also described in unicellular organisms, including eukaryotic microalgae (Barreto Filho et al., 2022; Berges and Choi, 2014; Darehshouri et al., 2008; Yordanova et al., 2013; Zuppini et al., 2010). However, although the major toxicological concern about the contaminants is related to their detrimental effects at individual and population levels on exposed aquatic organisms, the activation of PCD related-phenomena as a potential harmful consequence of contaminants exposure is still underexplored, especially on microorganisms such as microalgae (Bidle, 2016; González-Pleiter et al., 2017; Esperanza et al., 2017).

In the previous work (Esperanza et al., 2019), the most altered parameter for both UV-filters was intracellular hydrogen peroxide production. Thus, the goal of the present work was to evaluate the occurrence of oxidative stress as well as PCD activation as potentially relevant mechanisms of toxicity of these emerging compounds on *C. reinhardtii*. To this end, a multibiomarker array has been tested at both cellular and molecular levels. To deepen the mechanisms which cause blockage of cell division, cellular proliferation was analysed, completing the study with the analysis of F-actin content, which is part of the mitotic spindle. Since chloroplast membrane perturbations were reported by Esperanza et al. (2019), analyses of the photosynthetic yield and the presence of cytoplasmic lipid droplets were also implemented. Triggering of intracellular free Ca^{2+} signalling was tested in the first hour of exposure, as a potential biomarker involved in the cellular stress response shortly produced after contaminant exposure. To study the damage provoked by ROS production, lipid peroxidation was also analysed as a confirmation of oxidative stress. In close relation to oxidative stress induction, the potential activation of PCD phenomenon was also proved through the analysis of caspase activity, DNA fragmentation through comet assay, and the presence of autophagic vacuoles. Finally, since Esperanza et al. (2019) results suggest that these emerging compounds can differently affect microalgal cells, the blockage of efflux pumps, which serve for the active transport of xenobiotics to the outside of the cell has also been studied. To the best of our knowledge, this is the first work that tests this parameter as a potential biomarker in toxicity assays with microalgae.

2. Materials and methods

2.1. Microalgal species, contaminants and experimental setup

Chlamydomonas reinhardtii (strain CCAP 11/32A mt+) was cultured following the conditions of culture medium, agitation, and illumination detailed in Esperanza et al. (2019). in Tris-minimal phosphate medium (Harris, 1989) on a rotary shaker set at 150 rpm, under controlled conditions: 21±1°C and illuminated with 100 μmol photonm^{-2} s^{-1} under a 12:12 h light:dark cycle.

The compounds tested in this work were BP-3 (2-hydroxy-4-methoxybenzophenone) and BP-4 (5-benzoyl-4-hydroxy-2-methoxybenzenesulfonic acid), with purity higher than 97%, both obtained from Sigma-Aldrich. The structure and some physicochemical properties of BP-3 and BP-4 were detailed in Table S1.

Microalgal cells in the exponential growth phase adjusted to a cell density of 2×10^5 cells mL^{-1} were exposed to the UV-filters for 24 h. Exposures were performed in triplicates in 250 mL glassware flasks filled with 50 mL of microalgal culture under the same environmental conditions as stock cultures. Each benzophenone was conveniently resuspended in methanol to obtain the desired final concentrations, adding the same amount of methanol in all treatments, namely, the 0.02% (v/v) of final culture volume. No significant differences have been described in the growth of these microalgal cultures without and with 0.02% methanol. Based on this, cultures with methanol were always used as control ones in all assays (Esperanza et al., 2019).

Selected concentrations of both BPs were previously tested by Esperanza et al. (2019) considering the EC_{50} values for growth inhibition at 96 h (Table 1) and expressed in toxic units (TU; Bundschuh et al., 2014).

The concentrations of BP-3 and BP-4 corresponding to 0.5, 1 and 2 TUs were used in all the experiments (Table 1). Triplicate cultures were tested for each assayed concentration as well as for the control ones. Effective EC_{50}-concentrations of BP-3 and BP-4 at 0 and 24 h were confirmed by a HPLC–MS analysis (Table S2). Biomarkers assays were performed after 24 h of exposure, except in the case of two parameters: intracellular free Ca^{2+} concentration (analysed 1 h after exposure) and F-actin content (analysed 8 h after exposure).

2.2. Flow cytometry protocols

FCM methods were conducted using a Gallios flow cytometer (Beckman Coulter Inc.). This system is equipped with 2 lasers (488 nm and 633 nm), forward- (FSC) and size-scatter (SSC) light detectors, and fluorescence channels corresponding to the FL1 (505-545 nm), FL2 (560-590 nm), FL3 (605-635 nm) and FL4 (660-700 nm) spectral bands. Excitation laser at 488 nm was used as source of illumination in all assays, and 10,000 cells per replicate were analysed. Microalgal population was characterized in the cytometer by its size (FSC) and chlorophyll *a* content (red autofluorescence, FL4 detector). All protocols for the different biomarkers were pre-validated using positive controls to determine the areas and regions chosen for the final assays. A description of the flow cytometry methods and the fluorochromes used was summarised in Table S3. Data were analysed with the Kaluza Flow Cytometry Analysis V.1.1. software (Beckman Coulter Inc.).

Unless otherwise specified, cultures samples were counted and adjusted to a density of 2×10^5 cells mL^{-1} in phosphate buffer (PBS). Cells were marked with each probe in dark and at 20°C.

Table 1
EC_{50}, tested concentrations, and equivalent TU values (Esperanza *et al.*, 2019).

Contaminants	EC_{50} (mg L^{-1})	Assayed concentrations (mg L^{-1})	Toxic units (TU)
BP-3	5	2.5; 5; 10	0.5; 1; 2
BP-4	38	19; 38; 76	0.5; 1; 2

2.2.1. Cell proliferation

For the quantitative study of cell division, the intracytoplasmic dye 5-6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich) was applied as described in Rioboo et al. (2009). Before chemicals exposure, cells were marked with 4 μg mL^{-1} of CFSE for every 150 × 10^6 cells for 40 min. Fluorescence was collected in the FL1 detector and results were expressed as the population of daughter cells.

2.2.2. Intracellular lipid droplets

Fluorochrome Nile Red (NR; Sigma-Aldrich) was used to detect cytoplasmic lipid accumulations inside cells. These reserves are mainly composed of neutral lipids, usually triacylglycerides and cholesterol esters (Martin and Parton, 2006). Cells were dyed with 0.25 μg mL^{-1} of Nile Red for 5 min. The emitted fluorescence was collected in the FL2 detector and results were expressed as cells with lipid droplets (% NR +).

2.2.3. F-actin content

To determine alterations in the formation of F-actin in the cytoskeleton of microalgae exposed to BP-3 and BP-4, an analysis using phalloidin conjugated with fluorescein-isothiocyanate (FITC-phalloidin) was carried out by FCM. Phalloidin is a peptide obtained from *Amanita phalloides* which allows the selective labelling of F-actin. The FITC-phalloidin molecule applied exhibits a maximum excitation/emission at 496/520 nm (Endresen et al., 1995). *C. reinhardtii* cells (10^6) were fixed with 10% paraglutaldehyde in PBS, for 15 min on ice. Then, cells were washed in PBS, and incubated with FITC-phalloidin at a final concentration of 0.5 μg mL^{-1} for 30 min. The emitted fluorescence was collected in the FL1 detector. Results were expressed as cells with an increase in the F-actin content (% FITC +).

2.2.4. Intracellular Ca^{2+} levels

Calcium Green-1 acetoxymethyl ester (Calcium Green-1 AM; Thermo Fisher) fluorochrome was used to detect changes in intracellular levels of free Ca^{2+} (Prado et al., 2012). Cells were dyed with 10.32 μg mL^{-1} of fluorochrome for 2 h at 25°C (Esperanza et al., 2017). The fluorescent emission was collected in the FL1 detector and results were expressed as cells that present an increase in intracellular calcium levels (% Ca^{2+} +).

2.2.5. Lipid peroxidation

Image-iT™ Lipid Peroxidation (Thermo Fisher) was used for the detection of lipid peroxidation in *C. reinhardtii* cells. This product contains BODIPY® 581/591 C11 reagent, which binds to lipids of cellular membranes. Upon oxidation by lipid hydroperoxides, which are the final products of lipid peroxidation (Gaschler and Stockwell, 2017), this compound displays a shift in fluorescence emission from 590 nm to 510 nm. Therefore, the 510 nm/590 nm fluorescence ratio is directly proportional to the degree of lipid peroxidation of the cells. The emitted fluorescence is collected in FL1 and FL3 detectors so an increase in the ratio between FL1/FL3 indicates an increase in lipid peroxidation. Microalgae were marked with 10 μM of the dye for 30 min. Finally, cells were centrifuged and resuspended in PBS. Results were expressed as cells that show lipid peroxidation (% Peroxidation +).

2.2.6. Caspase activity

To detect the potential activation of caspase activity in microalgal cells exposed to UV-filters, the compound CellEvent Caspase-3/7 Green (Thermo Fisher) was used, as described in Esperanza et al. (2017) and González-Pleiter et al. (2017). Culture samples were treated with the reagent for 1 h at 25°C. The emitted fluorescence was collected in the FL1 detector and results were expressed as cells with an increase in caspase activity (% Caspase +).

2.2.7. Blockage of efflux pumps

Efflux pumps serve for the active transport of xenobiotics to the outside of the cell, so they constitute a mechanism of multixenobiotic resistance (MXR). But some compounds have been shown to block the activation of these efflux pumps (Luckenbach and Epel, 2005; Smital and Kurelec, 1998). To this aim, the elimination rate of fluorochrome rhodamine 123 (R123; Sigma-Aldrich) was analysed. R123 penetrates by passive diffusion to the cell. The efflux of rhodamine is carried out by the transport glycoprotein P that is one of the extrusion pumps (Smital and Kurelec, 1998). R123 has a maximum excitation/emission at 505/525 nm when ethanol is used as a solvent (Feller et al., 1995; Forster et al., 2012). *C. reinhardtii* cells were marked with 400 ng mL^{-1} of R123 for 30 min. In order to verify that R123 has entered the cells, they were analysed in the cytometer, always showing an increase in fluorescence of 100% in the microalgal population. Then, cells were centrifuged to remove the R123 present in the medium and resuspended in PBS. After 5 min, they were re-analysed in the cytometer to study the percentage of cells that expel the fluorochrome. The emitted fluorescence was collected in the FL1 detector and results were expressed as cells that expel R123 (% R123 -).

2.3. Photosynthetic yield

Changes in effective quantum yield (QY) in photosystem II (PSII) in *C. reinhardtii* cultures was analysed by Pulse Amplitude Modulation fluorometry with an AquaPen-AP-C100 fluorometer as described in Seoane et al. (2019).

2.4. Comet assay

Genotoxic effects of the benzophenones on *C. reinhardtii* were analysed by the detection of breaks in the DNA strand using comet assay as described by Prado et al. (2009) and Esperanza et al. (2015). For each preparation, 100 cells were analysed by fluorescence microscopy. Results were expressed as the percentage of DNA present in the tail of the comets (% tDNA) that was analysed using the OpenComet plug-in of the ImageJ software.

2.5. Autophagic vacuoles

Monodansilcadaverine (MDC; Sigma-Aldrich) was used to detect the presence of autophagic vacuoles as described in Esperanza et al. (2017). MDC was added to cell suspensions at a concentration of 16.8 μg mL^{-1} for 10 min. For each preparation, 100 cells were analysed by fluorescence microscopy, and results were expressed as cells with autophagic vesicles (% MDC +).

2.6. Data analysis

Results were normalised to the corresponding control, which was consider as 0. For each experiment, the hypothesis that the concentration of the tested compound does not affect the parameter was analysed by an ANOVA using SPSS Statistics software (v. 25.0, SPSS, IBM). When the hypothesis was rejected, Tukey's *post hoc* test was used to analyse how each concentration of benzophenone affects the parameter studied and to group the different treatments, with p-value < 0.05.

Graphs were made with the advanced graphics package *SigmaPlot v. 12.0*. Representative cytograms, created using the *Kaluza software version 1.1* (Beckman Coulter Inc.), are shown in Supplementary Material (Fig. S1-S7).

3. Results and discussion

3.1. BPs block cell proliferation and photosynthetic process altering energy metabolism

After 24 h of exposure, results showed a negative effect of these benzophenones on the cell proliferation of *C. reinhardtii*. A remarkable decrease ($p < 0.05$) in the population of daughter cells was observed in cultures treated with both pollutants (Figs. 1 and S1). These

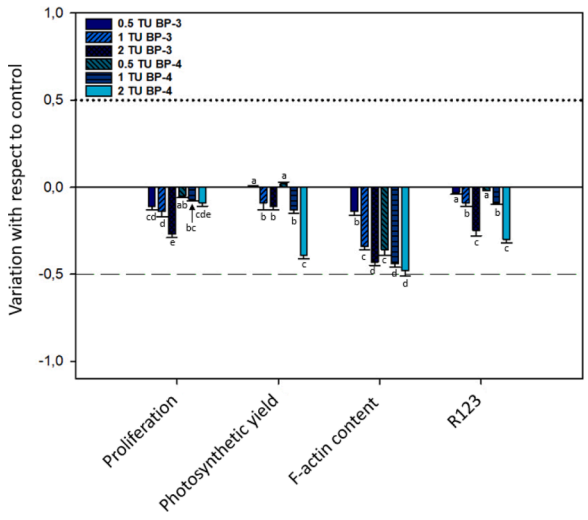

Fig. 1. Variations in cell proliferation, photosynthetic yield, F-actin content and in the percentage of cells that expel R123 on *C. reinhardtii* cells after 24 h-exposure to BP-3 and BP-4 (0.5, 1 and 2 TU), except for the F-actin content assay, which was measured after 8 h of exposure. Data are shown as relative response with respect to the control (0). Letters indicate significant differences ($p < 0.05$) between treatments according to the Tukey's *post hoc* test, except for letter *a*, which indicates that there are no significant differences between treatment and control. Dashed lines indicate reduction or increase by 50% with respect to the control.

benzophenones affected cell proliferation in a concentration-dependent manner, but the decrease was more pronounced in BP-3-exposed cultures. In these treatments, proliferation decreased by 11%, 14% and 27% in 0.5, 1 and 2 TU, whereas the exposure to BP-4 decreased cell proliferation by 5%, 7% and 9% in 0.5, 1 and 2 TU (Fig. 1). These results agree with those obtained for population growth (Esperanza et al., 2019), since it was observed that BP-3 also affected growth rates more markedly than BP-4. Although there are few previous studies where this parameter was analysed, a decrease in the cell proliferation has also been observed by CFSE staining in *Chlorella vulgaris* treated with an herbicide (Rioboo et al., 2009).

Moreover, possible alterations in the photosynthetic process caused by these emerging contaminants were analysed. Esperanza et al. (2019) reported alterations on the chlorophyll *a* fluorescence of cells exposed to both BPs. To delve into these results, QY of PSII was assessed. A remarkable decrease ($p < 0.05$) in the photosynthetic yield was detected after 24 h of exposure to 1 and 2 TU of both UV-filters, in a concentration-dependent manner (Fig. 1). This decrease was more pronounced at the highest concentration of BP-4, where the photosynthetic yield decreased by 39% (Fig. 1). Considering these results, it can be inferred that these compounds caused photosynthetic blocking. Photosynthesis-related processes as a toxic mechanism of different pollutants (triclosan, benzophenones, selenate, nanoplastics…) were assessed (Almeida et al., 2017; Cid et al., 2012; Esperanza et al., 2019; Geoffroy et al., 2007; Huang et al., 2018; Juneau et al., 2002; Sendra et al., 2019; Seoane et al., 2017). Therefore, photosynthesis seems to be a general toxicological target for contaminants in microalgal cells, which would finally alter the CO_2 fixation cycle in aquatic ecosystems. Since the rise in atmospheric CO_2 levels is now a serious threat because of its role in global warming (IPCC, 2018), the impact of these compounds on the behaviour of carbon fixing organisms should be considered as a high priority scientific goal in future ecotoxicological investigations.

The energy imbalance experienced by microalgal cells when they are exposed to an external stress factor, e.g., lack of nutrients or oxidative stress, could trigger the accumulation of neutral lipids (Gastaldi et al., 2007; Hu et al., 2008; Solovchenko, 2012; Zienkiewicz et al., 2016). Exposure to BP-3 induced a noticeable rise ($p < 0.05$) in the population of cells with accumulation of lipids on *C. reinhardtii*, showing an increment of cells that present lipid droplets of 0.32-, 0.99- and 2.11-fold in 0.5, 1 and 2 TU (Figs. 2 and S2). Lipid accumulation had already been described under exposure to emerging pollutants like nanoparticles in *Scenedesmus sp.* and *Thalassiosira sp.* (He et al., 2017; Pham, 2019). This increase in cytoplasmic lipid accumulations in cells exposed to BP-3 could be in close connection with an energy imbalance in microalgal cells, leading to the synthesis of neutral lipids, and finally blocking the normal cell division cycle (Fig. 1). In fact, alterations in the mitochondria of *C. reinhardtii* were previously observed by Esperanza et al. (2019), where a hyperpolarization in cells exposed to BP-3 and a depolarization in cells exposed to BP-4 were observed. These results reinforce the hypothesis of severe disturbances on the global energy status of *C. reinhardtii* exposed to both UV-filters.

3.2. BPs inhibit extrusion pumps

In response to exogenous compounds, eukaryotic and prokaryotic cells can activate several multixenobiotic resistance (MXR) mechanisms. In particular, the activation or inhibition of extrusion pumps in cytoplasmic membranes constitutes one of the most frequently studied MXR mechanisms in pharmacological assays. The role of these extrusion pumps is the active transport of xenobiotics to the outside of the cell (Bard, 2000; Cornwall et al., 1995) but, conversely, a blockage of them could exacerbate the toxic impact exerted by chemical compounds on exposed cells. However, this parameter is rarely considered as a potential exposure endpoint in toxicity assays with aquatic contaminants. Results showed that exposure to both UV-filters induce a strong inactivation of the extrusion pumps in *C. reinhardtii*; whereas in control cultures, all the microalgal cells were able to efficiently expel the fluorochrome from their interior. A relevant drop ($p < 0.05$) in the population with MXR activity was recorded in cultures exposed to intermediate and higher concentrations of both contaminants (Figs. 1 and S3). In cells treated with BP-3 the percentage of cells that were able to expel R123 from inside decreased by 9% and 25% in 1 and 2 TU, and in BP-4-treatments, this percentage of cells decreased by 9% and 30% in 1

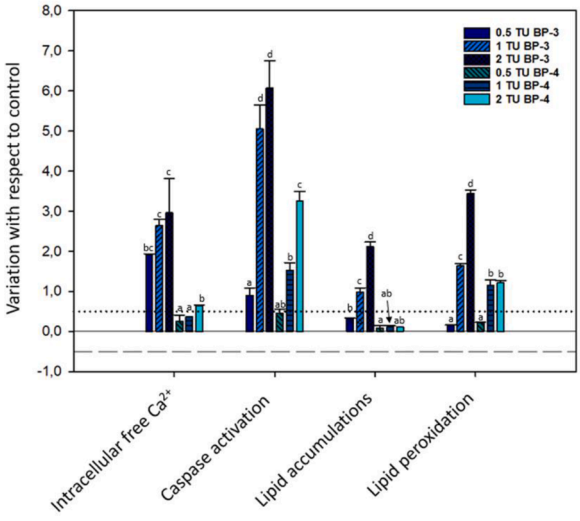

Fig. 2. Variations in intracellular free Ca^{2+} concentration, caspase activation, presence of cytosolic lipid accumulations and in lipid peroxidation on *C. reinhardtii* cells after 24 h-exposure to BP-3 and BP-4 (0.5, 1 and 2 TU), except for the free Ca^{2+} concentration test, which is measured after 1 h of exposure. Data are shown as relative response with respect to the control (0). Letters indicate significant differences ($p < 0.05$) between treatments according to the Tukey's *post hoc* test, except for letter *a*, which indicates that there are no significant differences between treatment and control. Dashed lines indicate reduction or increase by 50% with respect to the control.

and 2 TU, regarding to the control (Fig. 1). One of the hypotheses that could justify the difference in toxicity between the two benzophenones was the potential blockage of the efflux pumps, which serve for the active transport of contaminants outside cell, however, in this study a similar level of blockage of the efflux pumps was observed after the exposure to both sunscreens.

This phenomenon of blockage of extrusion pumps due to the exposure to UV-filters has been reported in the freshwater protozoan *Tetrahymena thermophila* (Gao et al., 2016). Results described in the present work also suggest that the inhibition of this MXR mechanism in unicellular organisms exposed to UV-filters can be part of the toxicity mechanism of these aquatic pollutants. Since this blockage of extrusion pumps could ultimately cause accumulation of UV-filters inside the cells, the study of this parameter can be especially useful to a better understanding of the deleterious damages provoked by these emerging pollutants in unicellular organisms such as *C. reinhardtii*.

3.3. BPs cause an increase in intracellular free Ca^{2+} and in lipid peroxidation

Calcium is a ubiquitous secondary messenger that participates in signal transduction in eukaryotic and prokaryotic cells (Sun et al., 2006; Wheeler et al., 2019). Population with high levels of intracellular free Ca^{2+} in microalgae exposed to all concentrations of BP-3 and to the highest concentration of BP-4 rose markedly ($p < 0.05$) (Figs. 2 and S4). After BP-3 exposure, the population with high intracellular free Ca^{2+} levels increased 1.92-, 2.65- and 2.96-fold in 0.5, 1 and 2 TU, while BP-4-treated cells, this percentage increased 0.65-fold in 2 TU, regarding to the control (Fig. 2).

In plant cells, abiotic stress or the exposure to nanoparticles or organic pollutants, leads to a sudden generation of free calcium in the cytoplasm due to the entry of Ca^{2+} from the extracellular space or due to its release from the endoplasmic reticulum or vacuoles (Bickerton et al., 2016; González-Pleiter et al., 2017; Prado et al., 2012; Sendra et al., 2019; Sun et al., 2006). In agreement with this, the increase in Ca^{2+} levels in cells exposed to BPs observed indicates that calcium also works as a cell signaller in the stress response caused by these UV-filters. Furthermore, since a relationship between Ca^{2+} and ROS levels has also been suggested (Gilroy et al., 2016), the increase in the concentration of this messenger in *C. reinhardtii* could be related to the alterations in the mitochondrial membrane potential, which can also trigger the overproduction of ROS observed by Esperanza et al. (2019). In this previous work, the presence of 0.5, 1, and 2 TU of BP-3, significantly increased the intracellular hydrogen peroxide levels in 1.9-, 2.1- and 2.3-fold; whereas in cultures exposed to 0.5, 1, and 2 TU of BP-4 results showed a significant increase of 1.2-, 1.4- and 1.5-fold, respectively, with respect to the control. Current results showed that the increase in ROS generation finally induces oxidative stress in *C. reinhardtii* cells as lipid peroxidation levels increased significantly ($p < 0.05$) after exposure to 1 and 2 TU of BPs (Fig. 2; Fig. S5). In BP-3-treatments, lipid peroxidation increased 1.64- and 3.44-fold in 1 and 2 TU, and in BP-4-treatments, lipid peroxidation increased 1.15- and 1.21-fold in 1 and 2 TU, with respect to the control (Fig. 2). Lipid peroxidation has been previously reported in the cyanobacterium *Microcystis aeruginosa* due to the exposure to BP-1 and BP-3, being more accused in BP-3 cultures (Mao et al., 2020). In the present work, results confirmed that the antioxidant system of the cell has been overcome, causing damage at cytoplasmatic membrane in *C. reinhardtii* exposed to BPs as already described by Esperanza et al. (2019), especially in cells exposed to BP-3. In addition, since neutral lipids, highly reduced compounds, can act as ROS neutralizers (Klok et al., 2013), their increase (Fig. 2) could be a consequence of the lipid peroxidation registered in cells exposed to both contaminants.

3.4. BPs induce PCD

PCD is a genetically determined and evolutionarily conserved process of self-destruction in eukaryotes and prokaryotes (Elmore, 2007). It can act as an adaptation mechanism for microalgal populations in unfavourable environmental conditions, such as the presence of toxic compounds, although there is still little information in the literature (Esperanza et al., 2017). Some of the hallmarks that characterize apoptotic cells are the loss of mitochondrial potential, activation of caspases, fragmentation of nuclear DNA and alteration of the cytoskeleton (Danon et al., 2000; Ly et al., 2003; Van Aken and Van Breusegem, 2015; Zuppini et al., 2010).

Actin is one of the main components of the cytoskeleton. This conserved eukaryotic protein participates in processes such as vesicle transport, endocytosis, cell mobility, and mitotic spindle formation (Doherty and McMahon, 2008; Onishi et al., 2016), being its principal role the formation of filaments (F-actin). The analysis of the possible alteration in the content of F-actin on *C. reinhardtii* was performed after 8 h of exposure to BPs, so that control cells no exposed to UV-filters were in active division process. A concentration-dependent decrease ($p < 0.05$) in F-actin content was recorded on *C. reinhardtii* cells exposed to all concentrations tested (Figs. 1 and S6). In microalgae exposed to BP-3, F-actin content dropped by 14%, 34% and 43% in 0.5, 1 and 2 TU, and in cultures exposed to BP-4, F-actin content dropped by 36%, 44% and 48% in 0.5, 1 and 2 TU (Fig. 1). This loss in F-actin content can explain the reported diminution in *C. reinhardtii* proliferation, since a decrease in this protein indicates an impossibility in the cytokinesis process on microalgal cells, blocking cell division at telophase (Harper et al., 1992; Yamagishi and Kawai, 2012).

Caspases are a set of proteases that mediate PCD, being in cells as inactive enzyme forms. Caspase-dependent PCD is activated by two ways, the extrinsic one, mediated by external signals and the intrinsic one, mediated by the mitochondria, both pathways converging in the activation of caspases (Elmore, 2007). *Chlamydomonas* exposed to 1 and 2 TU of BPs underwent a concentration-dependent increase ($p < 0.05$) in the population of cells with caspase activity (Figs. 2 and S7). In BP-3-treated cultures, caspase activity increased 5.05- and 6.07-fold in 1 and 2 TU, and in BP-4-treated cultures, it increased in 1.53- and 3.26-fold in 1 and 2 TU (Fig. 2). Since oxidative stress can produce changes in mitochondrial permeability (Van Aken and Van Breusegem, 2015), variations in mitochondrial transmembrane potential of *C. reinhardtii* exposed to BPs (Esperanza et al., 2019) could lead to the observed activation of caspases, as reported in other toxicity studies with this microalga (Esperanza et al., 2017; Yordanova et al., 2013).

Caspases are also directly related to the activation of endonucleases that cause degradation of nuclear DNA (Ulukaya et al., 2011). Comet assay results confirmed DNA fragmentation since the % tDNA in *Chlamydomonas* significantly ($p < 0.05$) increase in a concentration-dependent manner (Fig. 3). In BP-3-treatments, % tDNA increased 6.09-, 12.19- and 20.25-fold in 0.5, 1 and 2 TU, and in BP-4-treatments, it increased 14.30-, 14.40- and 20.96-fold in 0.5, 1 and 2 TU (Fig. 3). Similar results have been described in other studies with human keratinocytes exposed to BP-1 and with peripheral erythrocytes of the tropical fish *P. reticulata* exposed to BP-3, where an increase in % tDNA was also reported (Almeida et al., 2019; Amar et al., 2015), confirming the genotoxic damage provoked by these emerging compounds.

The alterations suffered by cells during PCD occur sequentially (Ulukaya et al., 2011; Van Aken and Van Breusegem, 2015). Thus, the observed DNA fragmentation could be in close relation to oxidative stress, reported as lipid peroxidation, decreased F-actin content and to the activation of caspase enzymes, which finally leads to activation of endonucleases.

3.5. BPs increase the percentage of cells with autophagic vacuoles

Autophagy is a lysosomal degradation pathway that contributes to

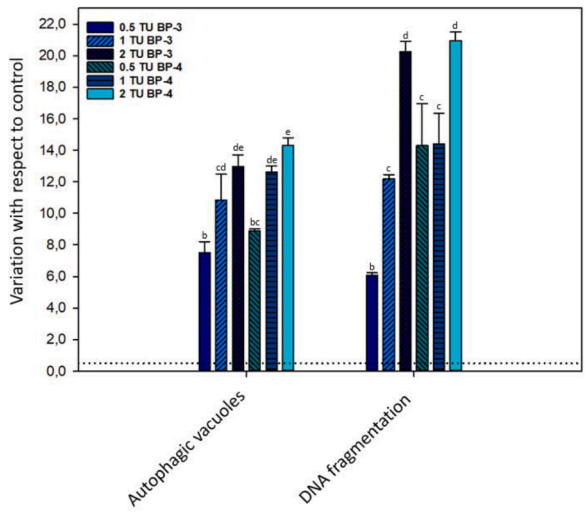

Fig. 3. Variations in the presence of autophagic vacuoles and DNA fragmentation on *C. reinhardtii* cells after 24 h-exposure to BP-3 and BP-4 (0.5, 1 and 2 TU). Data are shown as relative response with respect to the control (0). Letters indicate significant differences ($p < 0.05$) between treatments according to the Tukey's *post hoc* test, except for letter *a*, which indicates that there are no significant differences between treatment and control. Dashed lines indicate reduction or increase by 50% with respect to the control.

cell homeostasis through the function of autophagic vacuoles in the cytoplasm, allowing the removal of damaged membranes and organelles (Vicencio et al., 2008). There is a basal level of autophagy in cells and this level increases in stress conditions, such as oxidative stress or lack of nutrients (Pérez-Pérez et al., 2010, 2012). Although it has been traditionally considered as a class of PCD, recent studies suggest that its purpose is the degradation and recycling of cellular components, thus increasing viability, which is why it can also be considered as a mechanism of resistance to xenobiotics (Liu and Bassham, 2012). A concentration-dependent increase ($p < 0.05$) in the number of *C. reinhardtii* cells that present autophagic vacuoles in response to the exposure to all tested concentrations (Fig. 3). In BP-3-exposed cells, the presence of autophagic vacuoles increased 7.50-, 10.83- and 12.97-fold in 0.5, 1 and 2 TU, and in BP-4-exposed cells, it increased 8.90-, 12.61- and 14.33-fold in 0.5, 1 and 2 TU (Fig. 3). This increase was slightly greater in BP-4-treated microalgae and could be related to the lower lipid peroxidation and neutral lipid accumulation also reported in this work (Fig. 2). Based on this, this increase in the presence of autophagic vacuoles could be a potential recycling mechanism for membranes damaged by peroxidation. Therefore, the highest percentage of cells with autophagic vacuoles in BP-4 treatments, can support the greater viability of *Chlamydomonas* exposed to this compound (Esperanza et al., 2019). Similar results have already been observed in another study with *C. reinhardtii*, where an increase in the percentage of cells that presented autophagic vacuoles in response to the exposure to atrazine was also reported (Esperanza et al., 2017).

To summarize the effects of sublethal concentrations of BP-3 and BP-4 on the aquatic primary producer *C. reinhardtii*, a multibiomarker panel

Table 2
Summary of the response of *C. reinhardtii* to each biomarker assayed for each concentration of the BPs tested.

Biomarkers	BP-3			BP-4		
	0.5 TU	1 TU	2 TU	0.5 TU	1 TU	2 TU
Proliferation	↓	↓	↓		↓	↓
Photosynthetic yield		↓	↓		↓	↓
F-actin content	↓	↓	↓	↓	↓	↓
R123		↓	↓		↓	↓
Intracellular free Ca^{2+}	↑	↑	↑			↑
Caspase activation		↑	↑		↑	↑
Lipid accumulations	↑	↑	↑			
Lipid peroxidation		↑	↑		↑	↑
Autophagic vacuoles	↑	↑	↑	↑	↑	↑
DNA fragmentation	↑	↑	↑	↑	↑	↑

was carried out (Table 2). All the analysed parameters were altered in microalgal cells exposed to both BPs, except lipid accumulations for BP-4, in which the increase was not statistically significant. Since we are looking for sensitive biomarkers, focusing only on the lowest concentrations tested (0.5 TU), the altered parameters for BP-3 were cell proliferation, F-actine content, intracellular free Ca^{2+}, lipid accumulations, autophagic vacuoles, and DNA fragmentation (Table 2); whereas in the case of BP-4, the altered parameters were F-actine content, autophagic vacuoles, and DNA fragmentation (Table 2). Besides, the previous study reported that the lowest concentration of BP-3 significantly altered growth rate, chlorophyll *a* fluorescence, vitality, cellular metabolic activity, and ROS overproduction, whereas in the case of BP-4, 0.5 TU exposure only affected cellular metabolic activity, mitochondrial membrane potential, and intracellular ROS levels (Esperanza et al., 2019). Overall, in both studies, results suggest that BP-3 can be more harmful than BP-4 to aquatic ecosystems.

4. Conclusions

BP-3 and BP-4 blocked cell proliferation and caused a decrease in photosynthetic yield, which could indicate an alteration of the energy metabolism of the cell. Furthermore, it was verified that calcium can be acting as a signalling agent for the toxic impact generated by these contaminants. Besides, inhibition of extrusion pumps and appearance of oxidative stress were proposed as potential toxicity mechanisms for both compounds. Closely linked to this oxidative damage, the appearance of biomarkers related to the activation of PCD pathways was detected, including caspase activation, alterations in the cytoskeleton and DNA fragmentation. However, the detection of autophagic vacuoles could be part of a cell survival mechanism in *C. reinhardtii*.

The response pattern of *C. reinhardtii* was the same for both benzophenones, being PCD-related biomarkers, the most affected parameters, namely, alterations in the content of autophagic vacuoles and F-actin, DNA fragmentation, and caspase activation. Based on these data, the occurrence of PCD phenomena can provoke alterations in the microalgal populations, potentially compromising biodiversity, structure, and function of higher trophic levels in aquatic ecosystems.

CRediT authorship contribution statement

Laura Anido-Varela: Investigation, Formal analysis, Writing – original draft, Visualization. **Marta Seoane:** Methodology, Investigation, Formal analysis, Writing – review & editing, Visualization. **Marta Esperanza:** Methodology, Investigation, Formal analysis, Writing – review & editing, Visualization. **Ángeles Cid:** Resources, Funding acquisition, Writing – review & editing. **Carmen Rioboo:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106285.

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