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RESEARCH

Analysis of the effects of BP-3 and BP-4 on the transcriptome of *Chlamydomonas reinhardtii:* An RNA-Seq approach

Marta Esperanza[1] · Manuel Blanes-Rodríguez[1] · Ángeles Cid[1] · Marta Seoane[1]

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Abstract

Benzophenones (BPs) are one of the most widely used UV-filters and previous flow cytometric studies have shown that these aquatic emerging pollutants alter the physiology of the freshwater microalga *Chlamydomonas reinhardtii*. In order to obtain a more detailed study of the different cellular metabolic pathways affected, changes caused by BPs in the transcriptome of *C. reinhardtii* were investigated using RNA-Seq analysis after 24 h of exposure. Each benzophenone at its corresponding 96 h-EC_{50} value for growth provoked alterations in the gene expression of this microalga, although BP-3-exposed cells showed a higher number of differentially expressed genes than cells exposed to BP-4. GO enrichment analyses suggested that both compounds affected the same cellular metabolic pathways. Transcripts encoding for light-harvesting and chlorophyll-binding proteins were highly reduced. In addition, an overexpression of genes related to amino acid catabolism was also detected, suggesting that *C. reinhardtii* cells oxidize amino acids to obtain energy when photosynthesis was damaged by the pollutants. Regarding the oxidative damage provoked by the contaminants, genes encoding main antioxidant enzymes and involved in glutathione-associated metabolism were upregulated. Moreover, sulphur metabolism could have some relevance to explain the mechanism of action of BP-4 and its lower toxicity on microalgae, since the sulfonic acid group is the major structural difference between both BPs. Obtained results suggest that photosynthesis was impaired on cells exposed to the UV-filters, leading microalgae to obtain energy via a heterotrophic metabolism to survive. Thus, the occurrence of these sunscreens in freshwater ecosystems could trigger a worrying reduction in global CO_2 fixation.

Keywords Chlorophyta · Emerging pollutants · UV-filters · Gene Ontology · Photosynthesis · Heterotrophic metabolism

Introduction

Benzophenones (BPs) are organic compounds that work as chemical ultraviolet (UV)-filters, being able to absorb UV radiation. Awareness of the dangers of UV radiation exposure increased the utilisation of these chemicals in all types of cosmetic products to protect the skin from direct exposure to sunlight (Giokas et al. 2007). BPs are included in many personal care products (PCPs) such as sunscreens, lotions, and shampoos, but these compounds are even included in agricultural chemicals, plastic packages, or clothes, among others, as photostabilizer and sunblocking agents (Jeon et al. 2006). Due to their overuse, this kind of UV-filters are found in wastewater, rivers, lakes, and oceans worldwide (Balmer et al. 2005; Zhang et al. 2011; Rodil et al. 2012; Gago-Ferrero et al. 2013). Detected BPs concentrations are generally low in aquatic environments (ng L^{-1}); however, on sewage or recreational waters, depending on the location, the season or even the sampling conditions, BPs can reach levels of mg L^{-1} (Kim and Choi 2014; Tsui et al. 2014a, b; Careghini et al. 2015). Among BPs, benzophenone-3 (BP-3) and benzophenone-4 (BP-4) are the most frequently used and detected in environmental waters (Negreira et al. 2009; Rodil et al. 2012; De et al. 2013; Li et al. 2016). The effects of these UV-filters have been studied on different aquatic organisms, such as cyanobacteria, microalgae, clams, mussels, crustaceans, sea urchins, and fishes (Paredes et al. 2014; Petersen et al. 2014; Pablos et al. 2015; Du et al. 2017; Mao et al. 2017; Seoane et al. 2020; Yan et al. 2022), and it has been reported that both compounds induce biochemical, physiological, endocrine and genetic alterations (Diaz-Cruz and Barcelo 2009; Zhang et al. 2017; Meng et al. 2020; Carstensen et al. 2022; Colás-Ruiz et al. 2022).

✉ Marta Seoane
marta.seoane@udc.es

[1] Laboratorio de Microbiología, Facultad de Ciencias, Universidade da Coruña, Campus da Zapateira S/N, 15071 A Coruña, Spain

Microalgae are used as test organisms in ecotoxicity bioassays due to their ecological relevance and sensitivity. These microorganisms are primary producers, the base of the aquatic food chain, and any effect on them could affect higher trophic levels (Rioboo et al. 2007; Esperanza et al. 2020). In particular, the freshwater species *Chlamydomonas reinhartii* is also currently used in molecular studies as a biological model because its genome has been sequenced (Merchant et al. 2007). The potentially harmful effects of BP-3 and BP-4 have been reported on different freshwater and marine microalgal species (Sieratowicz et al. 2011; Petersen et al. 2014; Du et al. 2017; Mao et al. 2017; Seoane et al. 2017; Huang et al. 2018), however few works have studied in depth how these UV-filters alter cellular biological processes, since the toxic effects of these compounds on microalgae are usually evaluated using only growth inhibition tests. In previous studies, a multibiomarker panel using flow cytometry was carried out to evaluate the effects of BP-3 and BP-4 on the non-target microalga *C. reinhardtii* (Esperanza et al. 2019; Anido-Varela et al. 2022). Obtained results showed that sublethal exposure to both BPs blocks cell proliferation, increases intracellular free calcium, inhibits extrusion pumps, alters cytoplasmic and mitochondrial membranes and photosynthetic processes, and induces an increase in reactive oxygen species (ROS) production and lipid peroxidation. In relation to this oxidative damage, the activation of programmed cell death (PCD) pathways was detected, including caspase activation, alterations in the cytoskeleton, and DNA fragmentation.

In the field of ecotoxicology the use of transcriptomics has become a useful tool to assess the impacts of pollutants on organisms, providing accurate information on how xenobiotics affect microalgal cells at molecular and biochemical levels (Jamers et al. 2009, 2013; Esperanza et al. 2015, 2016; Qian et al. 2018; Duan et al. 2019). Therefore, the goal of this study was to investigate the transcriptomic alterations provoked by BP-3 and BP-4 on *C. reinhardtii* after 24 h of exposure using the RNA-Seq technique, delving into the previous results above mentioned (Esperanza et al. 2019; Anido-Varela et al. 2022). Differences in gene expression would allow a more detailed study of the different cellular metabolic pathways affected, being able to infer the mechanism of action of each compound on *C. reinhardtii*.

Materials and methods

Microalgal cultures, chemicals and experimental setup

Chlamydomonas reinhardtii (strain CCAP 11/32A mt+) was axenically cultured following the conditions of culture medium, agitation, and illumination detailed in Esperanza et al. (2019).

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 Suppl. Material 1 (Table S1). The 96 h-EC_{50} values for growth for BP-3 (5 mg L^{-1}) and BP-4 (38 mg L^{-1}) were selected for the experiments (Esperanza et al. 2019). In addition, to compare the response of the cells to the same sublethal concentration of these benzophenones, 5 mg L^{-1} of BP-4 was also tested. Each benzophenone was conveniently resuspended in methanol to obtain the desired final concentrations, adding the same amount of methanol in all treatments, the 0.01% (v/v) of final culture volume. No significant differences (*t*-test; p-value < 0.05) have been described in the growth of microalgal cultures with and without 0.01% methanol. Based on this, cultures with methanol were always used as control ones in the assays (Esperanza et al. 2019). Effective EC_{50}-concentrations of BP-3 and BP-4 at 0 and 24 h were confirmed by HPLC–MS (Suppl. Material 1; Table S2).

Exposures were carried out during 24 h in triplicate in flasks filled with 500 mL of culture under the same conditions as microalgal stocks. Microalgae in the exponential growth phase were used for the experiments, adjusting the cell density to 2×10^5 cells mL^{-1}. The cell density of each culture was obtained by counting culture aliquots on a Gallios flow cytometer. Then cells were harvested by centrifugation to obtain a pellet of 10^8 cells. Finally, samples were frozen with liquid nitrogen and preserved at -80 °C.

Total RNA extraction and RNA Sequencing

RNA isolation, library construction and sequencing were carried out by AllGenetics & Biology SL (www.allgenetics.eu) (Suppl. Material 1).

Data analysis

Raw reads in FASTQ format were analysed with FastQC software (Andrews 2010) and the adapters were trimmed with Trim Galore! (Martin 2011). These clean reads were then mapped to the reference genome (*C. reinhardtii* v5.5) using the splice-aware aligner Hisat2 (Kim et al. 2019). The reference genome and the Gene Transfer Format (GTF) files were obtained from Ensembl Plants (https://plants.ensembl.org/Chlamydomonas_reinhardtii/Info/Index). The aligned reads in BAM format were transformed into counts using HTSeq-counts (Anders et al. 2015) and the *C. reinhardtii* v5.5 GTF file for annotation.

Filtering and normalisation of the raw counts was performed using EdgeR R package (Robinson, et al. 2010; McCarthy et al. 2012) in order to do a differential expression analysis. For the filtering, a threshold of 0.5 CPM (counts per million) was used to remove low expressed genes. The normalisation technique used was TMM (Trimmed Mean of M-values) (Robinson and Oshlack 2010). Dispersion was estimated by quantile-adjusted conditional maximum likelihood included in EdgeR (Chen et al. 2014).

The differential expression analysis was carried out with the limma package (Ritchie et al. 2015) using the voom function (Law et al. 2014; Phipson et al. 2016). The *p*-values obtained were adjusted by the Benjamini-Hodchberg method (Benjamini and Hodchberg, 1995). For each comparison, genes with ($\log_2$FC > 0) were considered upregulated and genes with ($\log_2$FC < 0) were considered downregulated. An adjusted *p*-value of 0.05 was considered statistically significant.

Finally, a Gene Ontology (GO) analysis was conducted using the topGO R library (Alexa and Rahnenfuhrer 2020). The *C. reinhardtii* gene identifiers were retrieved using BiomaRt (Drost and Paszkowski 2017). A threshold of 0.01 was considered significant for the *p*-value obtained in the Fisher test applied in the comparisons. Hence the most represented terms in each ontology were selected. For graphic representation, -$\log_{10}$(*p*-value) was used in order to show the difference between the expression degree of each category.

Results and discussion

Differential gene expression

RNA-Seq data showed a total of 13,797 genes of *C. reinhardtii*. Results of differential gene expression between the different comparisons tested are summarized in Table 1.

After 24 h, results indicate that both UV-filters, in concentrations equivalent to their 96 h-EC_{50} values (5 mg L^{-1} for BP-3 and 38 mg L^{-1} for BP-4), produced alterations in the gene expression of *C. reinhardtii* (Table 1). But there was a noticeable variation in the number of differentially expressed genes between both treatments, suggesting that the metabolism of the microalga was more markedly altered in BP-3-exposed cells, in agreement with the results of our previous studies using flow cytometry (Esperanza et al. 2019; Anido et al., 2022). In fact, the number of upregulated genes in BP-3 (5 mg L^{-1}) compared to the control was more than double that in BP-4 (38 mg L^{-1}), and the number of downregulated genes in BP-3 (5 mg L^{-1}) was sevenfold higher than in BP-4 (38 mg L^{-1}) (Table 1). In addition, cultures exposed to 5 mg L^{-1} BP-4 did not show differences (*p*-value > 0.05) in gene expression regarding to the control. Since it was noticed that the concentration of 5 mg L^{-1} of BP-4 did not alter the transcriptome of microalgal cells, the comparisons of BP-4 (38 mg L^{-1}) *vs.* control, and BP-4 (38 mg L^{-1}) *vs.* BP-4 (5 mg L^{-1}) showed a similar number of altered genes (Table 1). These results could be graphically appreciated in Fig. 1. In view of these results, the comparisons BP-3 5 mg L^{-1} *vs.* control and BP-4 38 mg L^{-1} *vs.* control were used in the subsequent gene ontology analyses.

Gene ontology analyses

A GO analysis of the differentially expressed genes determined was carried out to classify them according to their functional characteristics in order to elucidate the underlying cellular mechanisms associated with the exposure to both BPs. For this purpose, two of the comparisons explained above (BP-3 5 mg L^{-1} *vs.* control and BP-4 38 mg L^{-1} *vs.* control) were selected to study the GO terms shared in both comparisons and the categories that were only enriched in each of the comparisons separately.

Shared GO terms for BP-3 and BP-4

GO terms related to photosynthetic processes were highly downregulated and stand out from the rest of the categories (Fig. 2). In the upregulated categories there are no GO terms that highlight from the others; however, grouping categories, it seemed that both compounds caused a markedly overexpression of genes related with the amino acid and glutathione metabolism and redox processes (Fig. 3). In this section we focused on some of the genes that were down- or upregulated for both compounds (Suppl. Material 2).

Table 1 Number of differentially expressed genes in *C. reinhardtii* cells. An adjusted *p*-value < 0.05 was considered statistically significant. For each comparison, genes with ($\log_2$FC > 0) were upregulated and genes with ($\log_2$FC < 0) were downregulated

	BP-3 (5 mg L^{-1}) *vs.* Control	BP-4 (5 mg L^{-1}) *vs.* Control	BP-4 (38 mg L^{-1}) *vs.* Control	BP-4 (38 mg L^{-1}) *vs.* BP-4 (5 mg L^{-1})
Upregulated	820	0	368	332
Not significant	12,046	13,797	13,300	13,357
Downregulated	931	0	129	108

Downregulated genes Generally, transcripts encoding for light-harvesting and chlorophyll-binding proteins and those associated with the carbon concentrating mechanism (CCM), or nitrate transport were downregulated. Thus, transcriptomic data suggested that photosynthesis might be impaired in microalgae exposed to the two BPs tested (Fig. 2).

a) Photosynthesis

Although a downregulation of genes related to photosynthesis and to chlorophyll biosynthetic processes was detected for both compounds, the most remarkable variations appeared in cultures exposed to BP-3 (Fig. 2). In fact, Anido-Varela et al. (2022) already reported that both BPs reduced the photosynthetic yield of *C. reinhardtii*; however, Esperanza et al. (2019) described a much higher increase in chlorophyll *a* fluorescence in cells exposed to BP-3 than in BP-4. A common mechanism of action was detected based on the obtained results and some genes related to the photosynthetic processes were downregulated after the exposure to both compounds: transcripts encoding Mg chelatase subunits (*CHLD, CHLI1* and *CHLI2*), chlorophyll binding proteins of the light harvesting complex II (*LHCA1, LHCA4* and *LHCA5*), PSI and PSII components (*PSAE, PSAK, PSAL, PSAI, PSBP4* and *PSBP6*) and RuBisCo (*RBCS1*) (Suppl. Material 2). Sunscreens are expected to affect the photosynthetic properties of exposed aquatic organisms, as they absorb UV rays, capturing

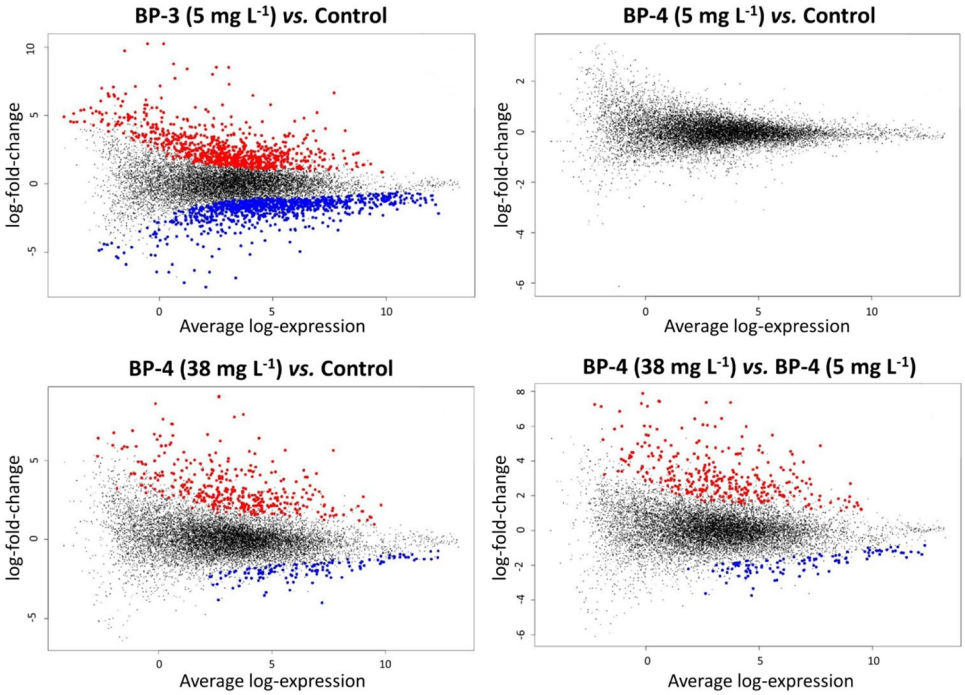

Fig. 1 Mean-difference plots (MD-plots) for each comparison. The Y-axis represents the log-fold-changes (log_2FC) and the X-axis represents the logarithmic mean expression. Each gene is represented with a dot. Differentially expressed genes (p-value < 0.05) are coloured in red (upregulated) and blue (downregulated)

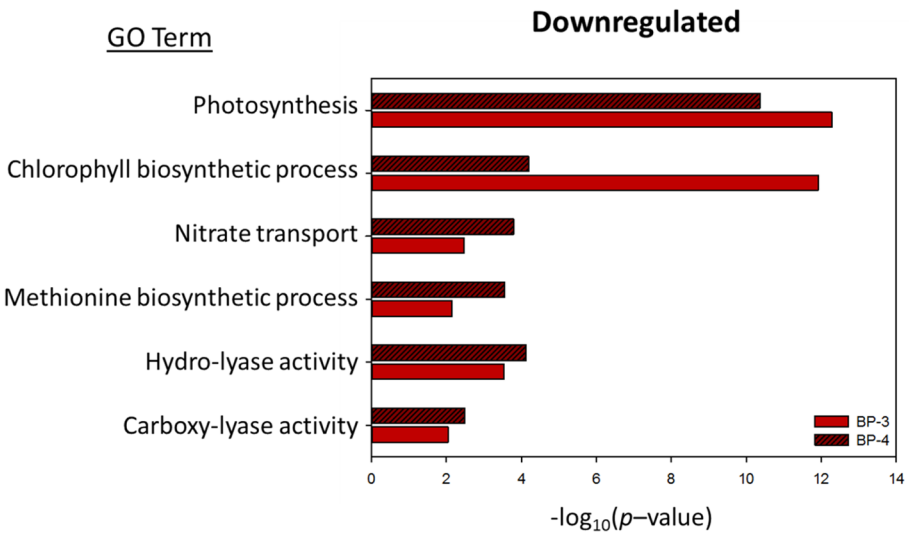

Fig. 2 Shared downregulated GO terms of differentially expressed genes in cultures exposed to BP-3 (solid bars) and BP-4 (dashed bars) for 24 h

incident energy and emitting it back as heat. UV-filters provoked alterations in photosynthetic activity, and one of the possible effects could be a drop in the cellular resources of NADPH and ATP that are produced in the light-dependent reactions (Kataria et al. 2014). To produce the necessary amounts of these energetic molecules, cells will need to reorganize their entire metabolism (Johnson and Alric 2013).

b) Nitrogen metabolism

UV-filters also alter nitrogen metabolism and a downregulation of transcripts related to high-affinity transport specific for nitrate and nitrate assimilation (*NRT2.2*, *NAR2* and *NAR1.6.*) was also detected after the exposure to both pollutants (Fig. 2; Suppl. Material 2). As BPs alter photosynthesis, they could also have an effect on the enzymes involved in nitrogen assimilation, as these two processes are linked and coordinated in microalgae (Prado et al. 2009). Furthermore, nitrogen assimilation in microalgae is a central pathway related to amino acid metabolism through nitrate reduction (Sanz-Luque et al. 2015).

c) Methionine biosynthesis

Transcripts related to methionine biosynthetic processes were also downregulated for both compounds (Fig. 2). These genes participate in chemical reactions and pathways resulting in the formation of methionine, a sulphur-containing essential amino acid found in peptide linkage in proteins. Indeed, *METE* transcript that encodes a 5-methyltetrahydropteroyltriglutamate-homocysteinem S-methyltransferase, involved in the first reactions that synthesizes L-methionine from L-homocysteine (Helliwell et al. 2014), was downregulated after UV-filters exposure (Suppl. Material 2).

d) Hydro-lyase and carboxy-lyase activities

The GO terms of hydro-lyase and carboxy-lyase activities were also downregulated (Fig. 2); they are implicated in the catalysis of the cleavage of a carbon–oxygen bond by elimination of water and the catalysis of the non-hydrolytic addition or removal of a carboxyl group to or from a compound, respectively. Several genes included in these GO categories were downregulated in cells exposed to the two BPs (Suppl. Material 2): *PEPC2* encodes a cytosolic phosphoenolpyruvate carboxylase*; UROD1* encodes uroporphyrinogen decarboxylase; *PGH1* encodes a phosphopyruvate hydratase; and *CAH1, CAH4* and *CAH5* encode carbonic anhydrases (CAs). CAs are important components of the CCM that increments photosynthetic productivity in microalgae by increasing levels of inorganic carbon (Gee and Niyogi 2017). Abundances of transcripts encoding CAs also decreased in previous studies with this microalgal species after the exposure to an herbicide (Esperanza et al. 2016) and in dark and anoxic conditions (Hemschemeier et al. 2013). It could be expected that the expression of these enzymes was downregulated since they are involved in the CCM, which exclusively works under light conditions (Tirumani et al. 2014). These results suggested again a change in microalgal cellular metabolism as reported in Esperanza et al. (2015).

Upregulated genes An overexpression of genes associated to amino acid degradation and redox misbalances was observed in both treatments. Increased amounts of transcripts linked to heterotrophic energy production suggested that the microalga *C. reinhardtii* oxidizes amino acids to obtain energy when photosynthesis was impaired by the pollutants (Fig. 3). These results were similar to those obtained in a prior transcriptomic study of the effect of atrazine on *C. reinhardtii* (Esperanza et al. 2015). Atrazine is an herbicide

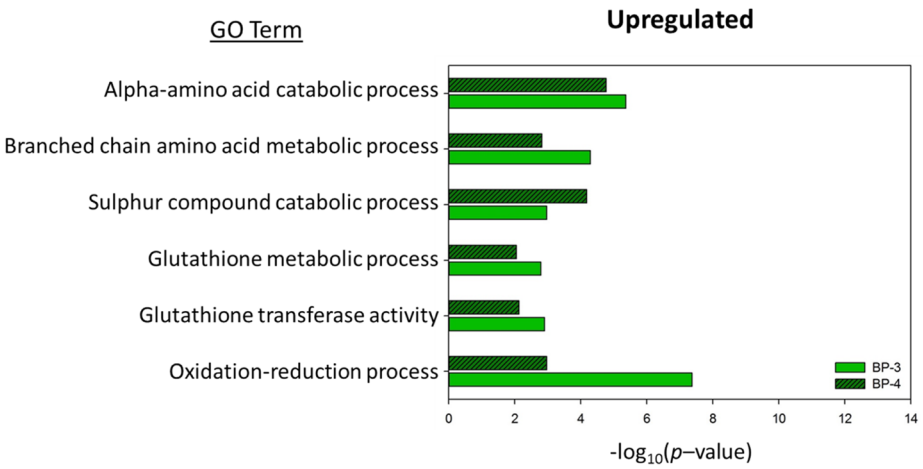

Fig. 3 Shared upregulated GO terms of differentially expressed genes in cultures exposed to BP-3 (solid bars) and BP-4 (dashed bars) for 24 h

whose mechanism of action impairs the photosynthetic apparatus. The decrease in photosynthetic activity and the positive regulation of cellular respiration showed that the effects produced by the BPs were very similar to those provoked by the herbicide.

a) Amino acid and sulphur compound metabolism

For both UV-filters, upregulated genes were significantly enriched with GO categories associated with amino acid and sulphur compound catabolism (Fig. 3) as *AAH1*, *IAD2, ALD8*, or *CDO2*, linked to the catabolism of the amino acids phenylalanine, tryptophan, valine or cysteine, respectively (Suppl. Material 2). Although most microalgae are photoautotrophic organisms, when autotrophic metabolism is not viable (photosynthesis inhibited), *C. reinhardtii* cells can grow under heterotrophic conditions, *i.e.*, using organic compounds as carbon source and without light (Morales-Sánchez et al. 2015; Zhang et al. 2019). Cells exposed to both BPs may stop cell division, break proteins down and oxidize amino acids, deviating the generated carbon skeletons to the tricarboxylic acid cycle (TCA), this leads to the accumulation of intermediates that can be utilised later for energy generation, as previously observed in the same microalgal species treated with atrazine (Esperanza et al. 2015). It was reported that changes in amino acid composition on microalgae can be used as sensitive biomarkers of stress (Chia et al. 2015). The expression of genes encoding for some enzymes related to amino acid metabolism was increased for both UV-filters, for example, the aromatic amino acid hydroxylase (*AAH1*) that catalyse the hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) (Vallon and Spalding 2009). The expression of *IAD2* gene was also enhanced and it encodes a hypothetical protein (tryptophan/indoleamine 2,3-dioxygenase-like enzyme) predicted to be involved in tryptophan catabolic process to kynurenines. In addition, the *AAD1* gene was upregulated, and it encodes an acetohydroxyacid dehydratase probably located in the chloroplast stroma and predicted to be implicated in the synthesis of valine, leucine, and isoleucine. Sulphur compound catabolic processes were also upregulated in BPs-exposed cells (Fig. 2). *CDO2* encodes a cysteine dioxygenase, an enzyme that oxidises the sulphur of cysteine (Cys), converting the thiol to cysteinesulphinic acid, an essential metabolite important for maintaining intracellular Cys homeostasis.

b) Glutathione metabolism

Upregulated genes were also notably enriched with GO terms related to glutathione metabolic processes for both pollutants (Fig. 3). BP-3 and BP-4 were identified as oxidative stress inducers in *C. reinhardtii* since high intracellular concentrations of H_2O_2 in treated cultures were previously reported by Esperanza et al. (2019) by flow cytometric analysis. Cellular detoxification is an essential physiological process that supplies defence against several environmental xenobiotics as well as against ROS generated by cellular metabolism, ensuring optimal conditions for cellular growth and survival (Islam et al. 2019). Glutathione-associated metabolism takes part in cellular detoxification, eliminating free radicals, reducing peroxides, or conjugating with electrophilic molecules (Hayes and McLellan 1999). The transcriptome of exposed microalgae indicated the presence of oxidative damage regarding the high levels of transcripts involved in glutathione metabolism such as those encoding for gamma-glutamyl transpeptidases (*GTP1*) or glutathione S-transferases (*GST2*) (Suppl. Material 2). *GTP1* is an integral component of membrane with peptidyltransferase activity and glutathione hydrolase activity, thus also involved in glutathione catabolic process. Glutathione S-transferases (GSTs) are a multifunctional protein family with catalytic properties and a detoxifying role, being a useful biomarker for the detection of oxidative damage induced by toxicants (Geoffroy et al. 2003; Nie et al. 2009; Esperanza et al. 2017). Furthermore, GSTs participate in several cellular mechanisms that regulate stress tolerance and in cell signalling processes (Chatzikonstantinou et al. 2017).

c) Oxidation–reduction processes

Regarding the positively regulated processes for both UV-filters, we also found terms related to oxidation–reduction processes (Fig. 3). Redox reactions are usually increased in cells in the presence of different toxic compounds since the oxidative stress provoked generate the overexpression of genes related to mitigating the presence of ROS through oxidation–reduction reactions. An increase in ROS is also expected as a result of the alteration of photosynthesis commented before. Thus, the expression of genes encoding main antioxidant enzymes was also enhanced in cells exposed to both compounds (Suppl. Material 2). For example, *MSD1* encode a manganese superoxide dismutase located in mitochondria and its induction was also detected in *C. reinhardtii* treated with heavy metals (Aksmann et al. 2014; Nowicka et al. 2016); *ALD5* and *ALD8* encode aldehyde dehydrogenases (ALDs), conserved NAD(P) + -dependent enzymes found in nearly all organisms which oxidise of reactive aldehydes. These molecules are implicated in several metabolic pathways such as glycolysis/gluconeogenesis (Yang et al. 2011; Brocker et al. 2013); but they could be harmful when their levels appeared in excess in cells. Thus, the activity of ALDs is relevant in the cellular equilibrium of aldehydes (Stiti et al. 2011; Tola et al. 2021), participating, for example, in the oxidation of aldehydes generated in lipid peroxidation processes (Zhao et al. 2017). Previous studies reported a great abiotic stress tolerance in *Arabidopsis* related with the upregulation of genes

encoding for ALDs (Zhao et al. 2017). In yeasts, *ALD5* is implicated in the synthesis of electron transport chain components of the mitochondria, being essential for respiration (Kurita 2003). Thus, the upregulation of *ALD5* in *C. reinhardtii* could be linked to an increment in the ATP generation by mitochondria. In previous studies, flow cytometric data reported an enhanced mitochondrial membrane potential of this microalga treated with atrazine and BP-3 (Esperanza et al. 2015, 2019). This hyperactivation of the mitochondrial activity reinforce the assumption that a considerable amount of energy may proceed from respiration. In addition, as commented before, the *C. reinhardtii ALD8* is implicated in the catabolism of valine and thymine.

The intracellular redox potential is also essential in cell growth regulation. *GLD1* was upregulated in microalgal cells exposed to the UV-filters and it encodes a Glucose-6-phosphate-1-dehydrogenase that catalyses the dehydrogenation of glucose-6-phosphate, the step 1 of the pentose phosphate pathway. Microalgal cells under the stress produced by exposure to BPs, could enhance the activity of GLD1 to increase NADPH production, an essential electron donor in the protection against oxidizing agents, thus trying to promote cell proliferation.

Another upregulated gene for both BPs related to the GO category of oxidation–reduction processes was *PTOX2* (Suppl. Material 2). It encodes a versatile plastid terminal oxidase in *C. reinhardtii* with physiological functions in chlororespiration, photosystem I (PSI) cyclic electron flow and carotenoid biosynthesis (Foudree et al. 2012). Chlororespiration is a process that can occur in the dark in the thylakoid membrane and in which electrons are transferred from NADPH to oxygen (Johnson and Alric 2013). One of the purposes of this process is to equilibrate the oxidation–reduction status of the plastoquinone (PQ) pool during cyclical flow of electrons around PSI (Trouillard et al. 2012) and PTOX2 prevents the PQ pool from over-reducing, by stimulating its reoxidation (Houille-Vernes et al. 2011). Cells might use chlororespiration to compensate for the synthesis of chemical energy when photosynthesis is inhibited by environmental stressors (Quiles 2006; Paredes and Quiles 2013).

Specific GO terms for BP-4

BP-4 provoked an upregulation in GO categories related to protein degradation and proteasome activity, response to hydrogen peroxide, cysteine metabolism and sulphate transport; whereas a downregulation was detected in GO terms related to arginine and glutamine metabolic processes, tricarboxylic acid metabolic pathways, FAD binding, oxidoreductase activity and secondary active transmembrane transporter activity (Fig. 4).

GO terms associated with proteolysis implicated in protein catabolism and peptidase and proteasome activities were upregulated in the presence of BP-4 (Fig. 4), which would reinforce the rise in the expression of genes related to protein and amino acid catabolism described previously. Some upregulated genes in these categories were *RPN7, RPN9*, *RPN10* and *RPN12* (Suppl. Material 2) that encode proteasome regulatory subunits implicated in ubiquitin-dependent protein catabolism. The proteasome is a big protein complex that degrades unnecessary or damaged proteins, representing an essential mechanism by which cells control the concentration of certain proteins. Proteasomal degradation is an important mechanism in several cellular processes, including gene regulation and oxidative damage response. In response to situations of cellular stress, such as abiotic changes and oxidative damage, there are cellular mechanisms to mark damaged proteins and promote their proteasomal degradation. Proper functioning of the proteasome can also help to maintain a correct photosynthetic activity in stressed cells. Indeed, an efficient proteasome activity in *C. reinhardtii* has been shown to be involved in the protection of PSII from photoinhibition (Mendoza et al. 2020). Vallentine et al. (2014) reported that slight selenite stress in *C. reinhardtii* promotes the activity of the ubiquitin–proteasome pathway (UPP), demonstrating that the activity of this protein complex increases in microalgae as a reaction to the stress. On the other hand, higher selenite concentrations drastically compromised the UPP activity, which was linked to ROS overproduction. Thus, the increased activity of the proteasome, only observed for BP-4 (which does not occur in BP-3), can be associated with the different level of stress that these compounds caused in cells. This is in agreement with the previous results (Esperanza et al. 2019; Anido-Varela et al. 2022), where it was reported that BP-3 caused more alterations in the physiology of the microalga than BP-4. Indeed, membrane integrity of BP-4-exposed cells remained intact; however, BP-3 exposure provoked a significant decrease in cell viability.

Upregulated genes were also significantly enriched with GO terms associated with the response to hydrogen peroxide. Most upregulated genes of this category encode small heat shock proteins (HSP22C, HSP22E, HSP22F) (Suppl. Material 2) that act as chaperones, being synthesized as a response to different environmental stressors and oxidative stress, not only during heat exposure, to protect proteins from induced damages and ensure the survival of cells. These proteins help fight acute stress when usual mechanisms for maintaining homeostasis are not enough. Microarray analysis performed with *C. reinhardtii* revealed that genes encoding HSP22E and HSP22F proteins are induced by oxidative stress (Fischer et al. 2005). Moreover, chloroplast small HSPs have been shown to protect PSII against oxidative stress in plant cells (Kim et al. 2012).

An increase in cysteine metabolism (cysteine catabolism and cysteine dioxygenase activity) was also detected (Fig. 4). During cysteine catabolism, this amino acid can be converted to pyruvate by different routes. Obtaining pyruvate may be related to the decrease in photosynthetic activity, which forces the microalgal cell to adapt its metabolism and produce ATP through other pathways such as cellular respiration. In González-Ballester et al. (2010) the transcript levels of CDO1 enzyme increased in *C. reinhardtii* cells during Sulphur deprivation and the activity of this enzyme was implicated with the reallocation of sulphur through the catabolism of cysteine and other molecules such as sulpholipids. The sulphate produced in cysteine catabolism could be transferred to biological molecules; in fact, sulphate transport was also highly induced in microalgal cells exposed to BP-4 (Fig. 4). Genes encoding SLT1 and SLT2 (Na^+/SO_4^{2-} co-tranporters), SULTR2 (H^+/SO_4^{2-} co-transporter) or SULP3 (a chloroplast sulphate transporter subunit) were overexpressed (Suppl. Material 2). The sulfonic acid group of the BP-4 molecule is the major structural difference between both UV-filters, so sulphur metabolism could have some relevance in explaining the mechanism of action of BP-4 and its lower toxicity on microalgae.

In most of the specific under-represented GO terms for BP-4, the same downregulated genes were detected (Fig. 4; Suppl. Material 2). For example, genes involved in the synthesis of arginine from glutamine via ornithine, *OTC1*, *AGS1* and *CMPS1,* were downregulated. The gene *NIT1* was also downregulated, and it encodes a nitrate reductase involved in nitrate assimilation. NIT1 expression is inhibited by ammonium, thus the downregulation of this gene could be due to the large amount of ammonium produced as result of amino acid catabolism. Nitrate reductase activity also decreased in *C. reinhardtii and C. moewusii* exposed to pesticides (Prado et al. 2009; Esperanza et al. 2015).

Specific GO terms for BP-3

In general, upregulated genes were significantly enriched with GO categories linked to tricarboxylic acid cycle and acyl-CoA metabolic process, peroxidase activity and cellular oxidant detoxification, and cytoplasmic microtubule; whereas downregulated genes were overrepresented with GO terms associated with ATP-dependent peptidase activity, ATPase binding, translation, thioredoxin peroxidase activity, glycolytic process, and fatty acid synthase activity (Fig. 5).

Processes associated with the mitochondria, the TCA cycle and acyl-CoA pathways were upregulated in cells exposed to BP-3 (Fig. 5). Glycolysis, TCA cycle, and mitochondrial respiration are crucial for cellular energy supply. Some genes upregulated for BP-3 were malate synthase (*MAS1*), one of the key enzymes of the glyoxylate cycle, an anabolic pathway

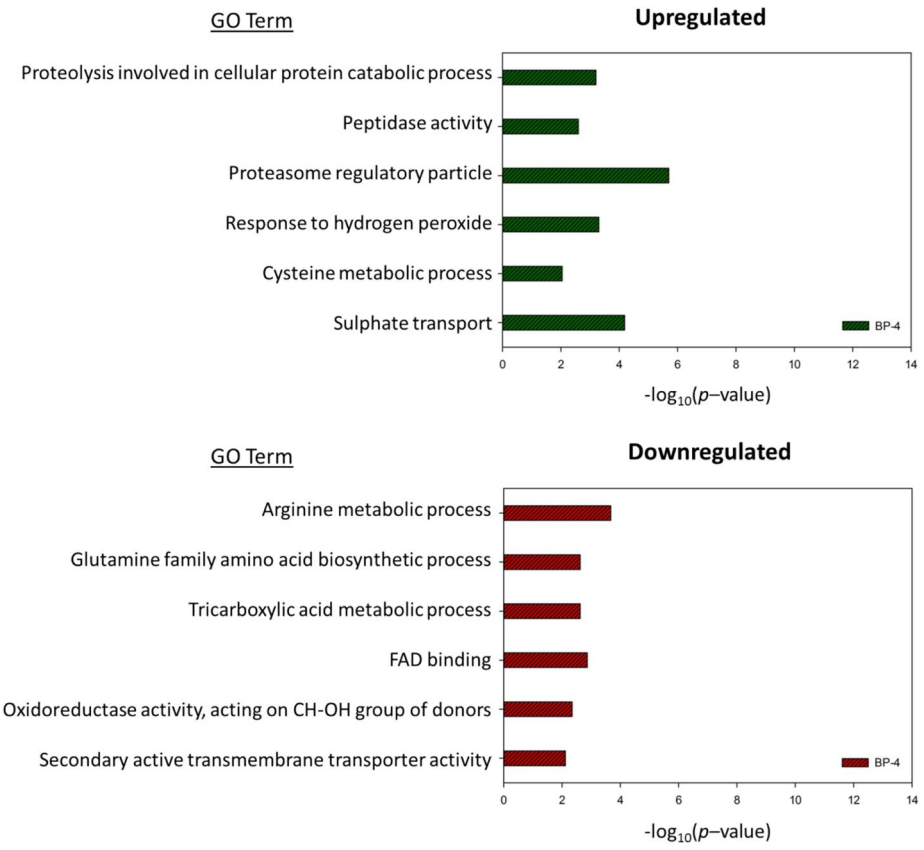

Fig. 4 GO analysis of differentially expressed genes in cultures exposed to BP-4 for 24 h. Upregulated (green) and downregulated (red) GO terms specific for BP-4

like the tricarboxylic acid cycle (TCA cycle) (Nogales et al. 2004), and *COX11, COX15, COX16* and *COX17* that encode cytochrome c oxidase assembly proteins (Suppl. Material 2). The cytochrome c oxidase participates in the last step of the mitochondrial respiratory chain, donating electrons to O_2 (Remacle et al. 2010). BP-3 also provoked an upregulation in the GO categories of peroxidase activity and cellular antioxidant detoxification. Peroxiredoxins (PRXs), such as PRX2 and PRX7 (Fig. 5; Suppl. Material 2), are essential scavengers of reactive oxygen and nitrogen species, but they are also involved in ROS signalling (Dayer et al. 2008).

In contrast, several ATP-dependent processes were downregulated, such as ATP-dependent peptidase activity and ATPase binding in general (Fig. 5). The photochemical phase of the photosynthetic process allows ATP synthesis by the ATP-synthases located on the thylakoid membrane. The inhibition of photosynthetic processes would lead, therefore, to an ATP deficit, which could explain the downregulation of ATP-dependent processes. Suppression of the photosynthetic activity would lead the cell to enhance other routes for obtaining ATP, such as cellular respiration. In this study *CLPR1, CLPR2, CLPR4, CLPR6, CLPP4* and *CLLP5* genes were downregulated (Suppl. Material 2). These genes encode caseinolytic protease proteolytic subunits (ClpP) that participate in maintaining organelle homeostasis (Zou and Bozhkov 2021), such as chloroplast ClpP, that play a key role in proteolytic pathways in *C. reinhardtii* (Derrien et al. 2012). In this study, the GO terms related to thioredoxin peroxidase activity were also downregulated (Fig. 5). Namely, chloroplast thioredoxins maintain the carbon fixation rate in photosynthesis through redox regulation (Schürmann and Jacquot 2000). It is noteworthy that peroxidase activity was upregulated as a response to the stress produced by BP-3; however, thioredoxin peroxidase activity was underexpressed. This may be related to the downregulation of transcripts with photosynthesis-related functions observed previously (Fig. 2) and in other studies with *C. reinhardtii* after the exposure to priority and emerging pollutants (Esperanza et al. 2016, 2019). Moreover, some genes implicated in fatty acid biosynthesis were identified, as 3-ketoacyl-acyl carrier protein synthase (*KAS2*) (Suppl. Material 2). Alterations in *KAS2* could be a signal of the beginning of cell's defence against lipid damage (Terashima et al. 2010).

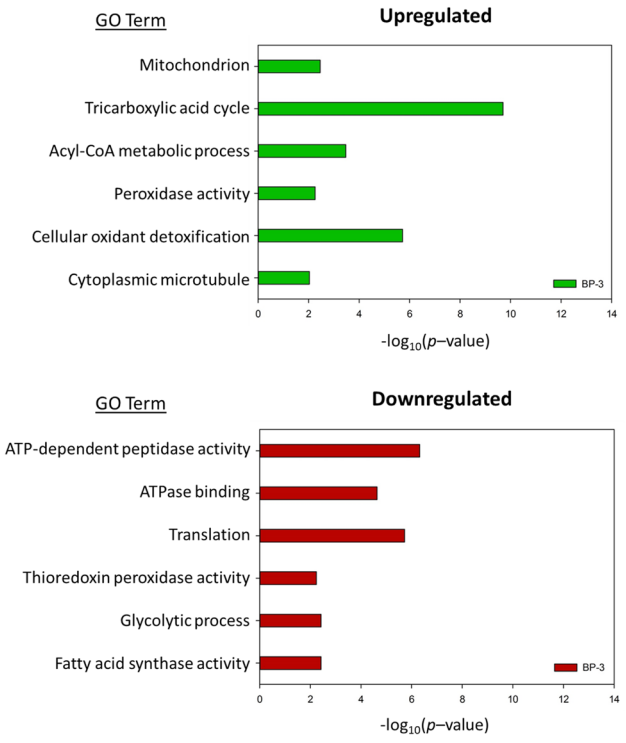

Fig. 5 GO analysis of differentially expressed genes in cultures exposed to BP-3 for 24 h. Upregulated (green) and downregulated (red) GO terms specific for BP-3

Conclusions

Each benzophenone at its corresponding 96 h-EC_{50} value (5 mg L^{-1} for BP-3 and 38 mg L^{-1} for BP-4) produced alterations in the gene expression of *C. reinhardtii*, but BP-3-exposed cells showed a considerably higher number of differentially expressed genes than cells exposed to BP-4. In general, transcriptomic data showed that both BPs caused similar metabolic changes in the microalga. Photosynthesis was altered in cells exposed to the UV-filters since transcripts encoding for light-harvesting and chlorophyll-binding proteins were highly diminished. In addition, genes encoding main antioxidant enzymes and involved in glutathione-associated metabolism were upregulated for both BPs as a response to the oxidative stress produced. Moreover, an overexpression of genes related to amino acid catabolism was also observed, suggesting that *C. reinhardtii* cells can grow under heterotrophic conditions, oxidizing amino acids to obtain energy when photosynthesis was impaired by the pollutants. This may have environmental consequences since the presence of these sunscreens in aquatic environments could trigger a reduction in global CO_2 fixation.

In general terms, the same cellular metabolic pathways were affected upon exposure to both BPs, and no differences were detected in the mechanism of action of each compound. However, GO categories related to proteasome activity were upregulated only in the presence of BP-4, and it seems that the slighter stress provoked by this compound may promote the proteasomal activity, while BP-3 could drastically impair its activity. Sulphur metabolism could also have some relevance to explain the mechanism of action of BP-4 and its lower toxicity on microalgae since the sulphonic acid group is the major structural difference between both UV-filters.

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Author contributions **Marta Esperanza:** Methodology, Investigation, Formal analysis, Writing—Original Draft, Visualization. **Manuel Blanes-Rodríguez:** Formal analysis, Data Curation, Writing—Original Draft, Visualization. **Ángeles Cid:** Conceptualization, Writing—Review & Editing, Supervision, Resources, Funding acquisition. **Marta Seoane**: Methodology, Investigation, Formal analysis, Writing—Original Draft, Visualization.

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Data availability Raw data generated during the current study that support the findings of this research are not publicly available but are available from the corresponding author under reasonable request. All derived data analysed during this study are included within this article and its supplementary material files.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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