ORIGINAL ARTICLE

Benzopyrene induces oxidative stress and increases expression and activities of antioxidant enzymes, and CYP450 and GST metabolizing enzymes in *Ulva lactuca* (Chlorophyta)

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Abstract

Main conclusion **Benzopyrene is rapidly incorporated and metabolized, and induces oxidative stress and activation of antioxidant enzymes, and CYP450 and GST metabolizing enzymes in *Ulva lactuca*.**

Abstract To analyze absorption and metabolism of benzo[*a*]pyrene (BaP) in *Ulva lactuca*, the alga was cultivated with 5 μM of BaP for 72 h. In the culture medium, BaP level rapidly decreased reaching a minimal level at 12 h and, in the alga, BaP level increased until 6 h, remained stable until 24 h, and decreased until 72 h indicating that BaP is being metabolized in *U. lactuca*. In addition, BaP induced an initial increase in hydrogen peroxide decreasing until 24 h, superoxide anions level that remained high until 72 h, and lipoperoxides that initially increased and decreased until 72 h, showing that BaP induced oxidative stress. Activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (AP), glutathione reductase (GR) and glutathione peroxidase (GP) were increased, whereas dehydroascorbate reductase (DHAR) activity was unchanged. The level of transcripts encoding these antioxidant enzymes was increased, but transcripts encoding DHAR remained unchanged. Interestingly, the activity of glutathione-S-transferase (GST) was also increased, and inhibitors of cytochrome P450 (CYP450) and GST activities enhanced the level of BaP in algal tissue, suggesting that these enzymes participate in BaP metabolism.

Keywords Benzopyrene · CYP450 oxidase · GST · Metabolism · Oxidative stress · Marine alga · *Ulva lactuca*

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed by benzene rings bonded in linear, clustered or angular arrangements (Abdel-Shafi et al. 2016; Mojiri et al. 2019). Small PAHs are constituted by two to four benzenes and large PAHs contain five and six benzene rings. The smaller PAHs is naphtalene, formed by two benzene rings, and phenanthrene (PHE) and anthracene (ANT) that are formed by three benzene rings. In particular, benzo[*a*]pyrene (BaP) is formed by six benzenes arranged as a cluster (Abdel-Shafi et al.

2016; Mojiri et al. 2019). PAHs are present in petroleum and they are produced by the incomplete combustion of organic materials such as wood, oils and coal or by anthropogenic cause such automobile emissions and cigarette smoking. PAHs are highly lipophilic and very soluble in organic solvents and they easily cross cellular membranes (Abdel-Shafi et al. 2016; Mojiri et al. 2019). PAHs are highly sensitive to light and heat, but they are resistant to corrosion. PAHs can be found in waters, sediments and soils and they are highly resistant to biodegradation, so they are biomagnified in the food chain. PAHs interfere with the function of membranes, and with proteins and enzymes located in intracellular membranes and they display mutagenic effects in animal and plants (Abdel-Shafi et al. 2016; Mojiri et al. 2019). In Chile, coastal zones have been impacted with successive spillages of crude oil in recent years affecting the health of population and the economy based on marine resouces, so it is urgently required to find biological tools to remove crude oil and PAHs from the environment.

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In animals, xenobiotics such as drugs and PAHs and endobiotics such as fatty acids, cholesterol, bilirubin and other lipids are detoxified through phase I and II metabolism (Jancova et al. 2010). Phase I reactions synthesize more polar compounds by addition of a hydroxyl group via membrane-bound cytochrome P450 (CYP450) that form an epoxide and cytosolic epoxyl-hydrolase form a dihydrodiol. Phase II reactions transform the hydroxylated compounds to a more excretable form by the addition of glucose, glucuronic acid, acetyl, sulfate, methyl, or glutathione groups through the action of glucosyltransferases, UDP-glucuronyltransferases, *N*-acetyltransfereases, sulfotransferases, methyltransferases and glutathione-*S*-transferases (GST), respectively (Jancova et al. 2010).

In terrestrial plants, as in animals, PAHs induce oxidative stress, activate antioxidant enzymes and metabolizing enzymes. In this sense, *Arabidopsis thaliana* plants cultivated with 1 mM PHE for 11 days showed inhibition of growth and the accumulation of hydrogen peroxide and lipoperoxides (Liu et al. 2009). In addition, *A. thaliana* plants cultivated with 0–1.25 mM PHE for 30 days showed an increase in activities of antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (AP) and catalase (CAT), and an increased synthesis of GSH (Liu et al. 2009). Moreover, *A. thaliana* plants cultivated with 0–1.25 mM PHE for 30 days showed a decrease in chlorophyll levels and the destruction of thylakoid and crista membranes in chloroplasts and mitochondria, respectively (Liu et al. 2009). The aquatic liverwort *Riccia fluitans* cultivated with 0.1–10 µM of PHE for 96 h showed an increase in hydrogen peroxide, lipoperoxides and carbonylated proteins indicating that an oxidative stress condition is induced by PHE (Burritt 2008). Furthermore, photosynthetic efficiency was decreased, chlorophyll and carotenoid levels were decreased, activities of antioxidant enzymes AP and GR were increased and the levels of ASC and GSH were enhanced (Burritt 2008). In addition, the levels of the polyamines spermine, spermidine and putrescine, which are involved in the protection of cellular and organellar membranes, increased as well as the activities of enzymes involved in polyamine synthesis (Burritt 2008). More recently, maize plants were cultivated in pots containing 1, 2.5 and 5% petroleum contaminated soils for 30 days (Cever-Keshin et al. 2018). A transcriptomic profile was obtained using microarray technique and it showed that the levels of transcripts of five GST, several cysteine proteases, involved in programmed cell death, and the activities of glutamine synthase, involved in N assimilation, and antioxidant enzymes SOD, AP, CAT and glutathione reductase (GR) were increased (Cever-Keshin et al. 2018).

Regarding microalgae and PAHs metabolism, the green microalga *Selenastrum capricornutum* cultivated 1160 µg L^{-1} of [^{14}C] BaP for 4 days metabolize BaP to 4,5 dBaP dihydrodiol and conjugate it with glucose or sulphate (Warshawski et al. 1990). Other green microalgae, *Scenedesmus acutus* and *Ankistrodemus braunii,* almost completely metabolize BaP and accumulate dihydrodiols whereas *Chlamydomonas reinhartdii*, *Ochromonas malhamenis* and *Euglena gracilis* did not metabolize BaP (Warshawski et al. 1995). In addition, the green microalga *S. capricornutum* cultivated with 266 ng mL^{-1} for 48 h metabolized BaP to dihydrodiols, mainly 4,5 dBaP, 7,8 dBaP and 9,10 dBaP (Hernández-Blanco et al. 2016). Regarding marine macroalgae and PAHs biodegradation, the green macroalgae *Ulva intestinalis* and *Chladophora glomerata* cultivated with BaP metabolized 42–49% of BaP in 6 h (Kirso and Ihra 1998). The metabolism of BaP in these green algae was dependent on CYP450, monooxygenase, dioxygenase, and peroxidase activities (Kirso and Ihra 1998). In contrast, the brown macroalgae *Fucus vesiculosus* and *Chorda filum* accumulate 89–99% of BaP but metabolized only 4% after 5 days. The brown macroalga *Laminaria japonica* cultivated with 0.1 mg L^{-1} of PHE and pyrene (PYR) for 14 days showed the accumulation and degradation of 90% of both PAHs (Wang and Zhao 2007).

The marine alga *U. lactuca* produced oxidized aromatic compounds such as polyphenols, alkaloids and oxidized and phlorotannins (De Wit et al. 2018). Phlorotannins act as a deterrent of grazing crustaceans, but they are not toxic for other herbivores such as gastropods (Peckol and Putnam 2016). In addition, extracts of *U. lactuca* contained oxygenated derivatives of unsaturated fatty acids indicating that this alga displays mechanisms allowing the transformation of non-polar molecules (Wang et al. 2013). Moreover, *U. lactuca* cultivated with sulfathiazole, an aromatic organosulfur drug, showed the absorption into algal tissue and partial degradation of the compound at 5 days of culture (Leston et al. 2014). The complexity of carbon metabolism in *U. lactuca* has been described as a strategy to survive episodes of desiccation and re-submersion in the intertidal zones to obtain energy from different carbon sources (Gupta and Kushwaha 2017). In addition, *U. lactuca* is a cosmopolitan species that has been found in the intertidal zones of central Chile, that are polluted with crude oil such as Ventanas and Quintero-Puchuncaví (Muñoz et al. 2020). Thus, *U. lactuca* constitute an interesting model to study the mechanisms involved in xenobiotic detoxification in marine algae. Results obtained regarding the mechanisms involved in PAHs degradation in *U. lactuca* will be useful for seawater phycoremediation of crude oil and PAHs in the future.

In this work, we analyzed the potential accumulation and metabolism of BaP, the induction of oxidative stress, the activation of the antioxidant and metabolizing enzymes in *Ulva lactuca*.

Materials and methods

Alga sampling

U. lactuca was collected from Cachagua (32°34′S, 71°27′W) a non-polluted site from central Chile, transported in a cooler at 4 °C, manually cleaned from other algae species, sonicated twice to remove epiphytic bacteria, and maintained in artificial seawater (Sigma-Aldrich, St Louis, CA, USA).

In vitro cultures

U. lactuca (10 g of fresh tissue) was cultivated in 300 mL of artificial seawater without (control) or with 1, 10, 50, 100 or 250 µM of benzo[*a*]pyrene (BaP) having a purity ≥ 99% (Sigma-Aldrich, St Louis, CA, USA) for 7 days, at 14 °C with air bubbling and a photoperiod of 14 h light and 10 h darkness. Since BaP has low solubility in water, a 25 mM of BaP stock solution was prepared in DMSO with a purity ≥ 99% (Sigma-Aldrich, St Louis, CA, USA) and then an aliquot was dissolved in the culture medium adjusting the DMSO to a final concentration of 0.5% v/v in the medium. These cultures were used to analyze the accumulation of BaP, for qPCR analyzes, the detection of enzyme activities, and only few fronds were used for cell viability analyzes. All these analyzes were performed as independent triplicates. For inhibition assays, 10 g of alga were cultivated in 300 mL of artificial seawater without (control) or with the addition of 200 µM of buthionine sulfoximine having a purity ≥ 99% (Royal Pharma, Hangzhou, China), which is an inhibitor of glutathione *S*-transferase, or with 25 µM of $CuSO_4$ having a purity ≥ 99% (Sigma-Aldrich, St Louis, CA, USA), which is an inhibitor of CYP450 (Letelier et al. 2009), and with 5 µM of BaP for 12 and 24 h, in triplicates.

Analysis of cell viability

Three algal lamina were cultivated without BaP and with increasing concentrations of BaP for 7 days and cell integrity and morphology were visualized using Axiovert 100 confocal microscope (Zeiss, Oberkochen, Germany) using an emission wavelength of 488 nm from an argon laser and a filter of 505–550 nm for detection of chlorophyll fluorescence. The images were analyzed using the software LSM510 (Zeiss, Oberkochen, Germany).

Extraction of benzopyrene from algae and seawater

The extraction of BaP from algal tissue was performed as described by Sadowska-Rociek et al. (2013), with modifications, and the extraction of BaP from artificial seawater was performed as described by Colombo et al. (2005), with modifications. One gram of algal dried tissue (DT), which corresponds to 7 g of algal fresh tissue (FT), were pulverized in a mortar with liquid nitrogen and homogenized in 15 mL of cyclohexane having HPLC grade purity (Merck, Darmstadt, Germany). Cellular debris was removed using Quechers AOAC method kit (Agilent Technologies, Santa Clara, CA, USA) and centrifugation at 10,000×*g* for 10 min. Pigments were removed using Quechers SPE dispersive kit (Agilent Technologies, Santa Clara, CA, USA) (Gratz et al. 2011). The extracts recovered (7 mL) were filtered using PVDF filters with 0.2 µm pore size (Finetech, Taichung, Taiwan) and stored in amber glass vials at 4 °C. The culture mediums (300 mL) were extracted twice with 200 mL of cyclohexane using an extraction funnel, and then the extracts were concentrated to 2 mL using a rotavapor HeiVap (Heidolph Industries, Schwabach, Germany). The extracts were filtered using PVDF filters with 0.2 µm pore size (Finetech, Taichung, Taiwan) and stored in amber glass vials at 4 °C.

Quantification of benzopyrene by HPLC

BaP in extracts was analyzed according to Gratz et al. (2011) using an Infinity 1260 series HPLC (Agilent Technologies, Santa Clara, CA, USA) having a reverse phase column Zorbax Eclipse XDB-C18 (4.5 mm × 15 mm and particle size of 5 µm, Agilent Technologies, Santa Clara, CA, USA) and a fluorescence detector. The elution program consisted in a mobile phase of H2O/CH3CN with a flux of 0.8 mL min^{-1} at 25 °C and a step from 0 to 1.5 min of 40% H_2O and 60% CH_3CN, a step from 1.5 to 7 min of 10% H_2O and 90% CH3CN, and a step from 7 to 13 min of 0% H_2O and 100% CH_3CN. BaP was detected using an excitation wavelength of 260 nm and an emission wavelength of 520 nm at a retention time of 9.6 min.

Quantification of hydrogen peroxide, superoxide anions and lipoperoxides

Hydrogen peroxide was determined as described in González et al. (2010). Algae (1.5 g of FT) were collected from cultures every 30 min and immediately incubated in 100 mM phosphate buffer pH 7.0 containing 10 µM of 2′,7′-dichlorodihydrofluorescein-diacetate (DCF-DA, Invitrogen, Carlsbad, CA, USA) for 30 min. Algae were rinsed with fresh artificial seawater and dried with tissue paper. The samples were pulverized in a mortar with liquid nitrogen, homogenized in 5 mL Tris–HCl pH 7.0 and centrifuged at 10,000×*g* for 15 min. DCF fluorescence was determined using an excitation wavelength of 480 nm and an emission wavelength of 590 nm using a spectrofluorometer PerkinElmer model LS-5 (PerkinElmer, Waltham, MA, USA) The concentration of

hydrogen peroxide was calculated using a calibration curve prepared with 0–1 μM of DCF.

Superoxide ions were determined as described in González et al. (2010). Algae (1.5 g of FT) were collected from cultures every 2 h and immediately incubated in 100 mM phosphate buffer pH 7.0 containing 100 μM hydroethidine (Invitrogen, Carlsbad, CA, USA) for 30 min. Algae were pulverized in a mortar with liquid nitrogen, homogenized in 5 mL Tris–HCl pH 7,0 and centrifuged at 10,000×g for 15 min. 2-hydroxy ethidium (2-HE) fluorescence was detected using an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a spectrofluorometer PerkinElmer model LS-5. The concentration of superoxide ions was calculated using the extinction coefficient of 2-HE ($\varepsilon = 9.4$ mM^{-1} cm^{-1}).

Lipoperoxides were determined as described in Ratkevicius et al. (2003). Algae (1 g of FT) were pulverized in a mortar with liquid nitrogen and homogenized with 5 mL of 0.1% v/v of trichloroacetic acid (TCA). The mixture was centrifuged at 10,000×g for 20 min, a sample of 200 μL was collected. The sample was mixed with 800 μL of an aqueous solution of 0.5% v/v thiobarbituric acid in 20% m/v TCA and the mixture was incubated at 90 °C for 30 min. The absorbance of malondialdehyde (MDA) adduct was measured at 510 nm using a spectrophotometer model Cary 8520 (Agilent Technologies, Santa Clara, CA, USA). The concentration of lipoperoxides was calculated using the extinction coefficient of MDA adduct ($\varepsilon = 155$ mM^{-1} cm^{-1}).

Preparation of protein extracts

Protein extracts were prepared González et al. (2010). Fresh tissue (6 g) was pulverized in a mortar with liquid nitrogen and homogenized with 30 mL of 100 mM sodium phosphate buffer pH 7.4 containing 5 mM 2-mercaptoethanol and the mixture was centrifuged at 10.000 rpm for 30 min at 4°. The proteins in the supernatant were precipitated with 0.6 g mL-1 ammonium sulphate, shaking at 4° for 1 h and then centrifuged at 10,000×g for 30 min. Protein pellet was solubilized in 1 mL 100 mM sodium phosphate buffer pH 7.4 containing 20% glycerol and 2 mM 2-mercaptoethanol. Protein concentration was determined according to Bradford (1976).

Detection of antioxidant enzyme activities

Superoxide dismutase (SOD) activity was determined in 1 mL of reaction mixture containing 30 mM Tris–HCl pH 7.0, 0.1 mM EDTA, 20 mM riboflavin, 0.6 mM NBT and without protein extract or with 50 μg of protein extract. The reaction mixture was incubated under white light for 15 min and absorbance of formazan, the reduced form of NBT, was determined at 560 nm. The specific activity was calculated considering as 1 U the amount of enzyme can reduce 50% of total NBT compared to control reaction mixture without the enzyme.

Catalase (CAT) activity was determined in 1 mL of reaction mix containing 100 mM sodium phosphate buffer pH 7.0, 1 mM hydrogen peroxide and without protein extract or with 30 μg of protein extract. The decrease in absorbance due to the consumption of hydrogen peroxide was detected at 240 nm for 2 min. The specific activity of CAT was calculated using a calibration curve prepared with hydrogen peroxide from 1 nM to 1 mM.

Ascorbate peroxidase (AP) activity was determined in 1 mL of reaction mixture containing 100 mM sodium phosphate buffer pH 7.0, 0.4 mM ascorbate, 1 mM hydrogen peroxide and without protein extract or with 20 μg of protein extract. The decrease in absorbance due to consumption of ascorbate was detected at 290 nm for 1 min. The specific activity was calculated using the molar extinction coefficient of ascorbate ($\varepsilon = 2.8$ mM^{-1} cm^{-1}).

Dehydroascorbate reductase (DHAR) activity was determined in 1 mL of reaction mixture containing 100 mM sodium phosphate buffer pH 7.0, 1 mM glutathione, 0.5 mM of dehydroascorbate, and 50 μg of protein extract. The increase in absorbance due to the production of ascorbate was detected at 290 was for 2 min. The specific activity was calculated using the molar extinction coefficient of ascorbate ($\varepsilon = 2.8$ mM^{-1} cm^{-1}).

Glutathione reductase (GR) activity was determined in 1 mL of reaction mix containing 100 mM sodium phosphate buffer pH 7.0, 0.5 mM GSSG, 0.15 mM NADPH and 20 μg of protein extract. The decrease in absorbance due to the consumption of NADPH was detected at 340 nm for 1 min. The specific activity was calculated using the NADPH molar extinction coefficient of NADPH ($\varepsilon = 6.2$ mM^{-1} cm^{-1}).

Glutathione peroxidase (GP) was determined in 1 mL of reaction mix containing 100 mM sodium phosphate buffer pH 7.0, 0.5 mM glutathione, 0.15 mM NADPH, 1 U glutathione reductase, 1 mM hydrogen peroxide and without protein extract or with 20 μg of protein extract. The decrease in absorbance due to the consumption of NADPH was detected at 340 nm for 2 min. The specific activity was calculated using the NADPH molar extinction coefficient of NADPH ($\varepsilon = 6,2$ mM^{-1} cm^{-1}).

Quantification of detoxification enzyme

Glutathione-S-transferase (GST) activity was determined in 1 mL of reaction mix containing 100 mM sodium phosphate buffer pH 7.0, 0.5 mM GSH, 1 mM CDNB and 50 μg of protein extract. The increase in absorbance due to the formation of the adduct GSH-CDNB was detected at 340 nm 2 min. The specific activity was calculated using the molar extinction coefficient of the adduct ($\varepsilon = 9.6$ mM^{-1} cm^{-1}).

RNA extraction and qPCR analysis

Total RNA was extracted from 50 mg of fresh tissue using the FavorPrep Plant Total RNA mini kit (Favorgen, Ping-Tung, Taiwan). Total RNA (2 μg) was used to synthesize cDNA using the Affinityscript qPCR cDNA synthesis kit (Agilent Technologies, Santa Clara, CA, USA). Real-time PCR was performed with 50 ng of cDNA, 400 nM of each primer and using Brilliant III ultra-fast SYBR green qPCR kit (Agilent Technologies, Santa Clara, CA, USA) and an Aria MX real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). The amplification program consisted of an initial step of 3 min at 95 °C and 40 cycles of 5 s at 95 °C followed by 10 s at 55 °C. Primers to detect SOD, AP, CAT, GR, GST transcripts were designed using sequences obtained from *U. intestinalis*, *U. fasciata* and *U. compressa* (NCBI GeneBank, *U. compressa* SRA records SRP145672 and PRJNA557176). Primers were: superoxide dismutase (GeneBank EF437244.1) SOD-F 5′CCT GCT CAG GCA ACC TCC TT3′, SOD-R 5′TGC CAG CCT TCC AGT CTC AC3′, catalase (GeneBank DQ286544.1) CAT-F 5′ACC AAG AGG CGG AGA AAG TG3′, CAT-R 5′CGA AGT CAA ACC GGT CCT CA3′, ascorbate peroxidase (GeneBank DQ286543.1) AP-F 5′CCA CAA GGC TGA GAC CAA GT3′, AP-R 5′CTT TGT CCA CTC AGG GGT CC3′, dehydroascorbate reductase (GeneBank XM_001698323.1) DHAR-F 5′GCG ACT CGT ACT GCT CCT AC3′, DHAR-R 5′GAA AAC GGA ACT CCG CGC TT3′, glutathione reductase (GeneBank DQ286546.1) GR-F 5′AGT TCG GGG ACG TGG ATG TG3′, GR-R 5′CTC TCG TTG CCG GAG ATC GT ‘3, glutathione peroxidase (GeneBank XM_001698523.1) GPX-F 5′CAC GCG TCC GGT AAC ACT3′, GPX-R 5′GGG ACG AGA AAG GTT GCT GA3′. Glutathione S-transferase (GeneBank FD387475.1) GST-F 5′GGA CCC ATG CAA CGC CAA G3′, GST-R 5′GGT GCG CTG TAC ACC CAA GA3′, and α-tubulin (GeneBank EU701065.1) as housekeeping transcript, TUB-F 5′ACA GGC TCA TTG CTC AGG TC3′, TUB-R 5′TCT CGG CAG AGA TGA CTG GA3′.

Statistical analysis

Experimental results were subject to one-way analysis of variance (ANOVA) and post hoc Tukey Multiple Comparison Test using the statistical software Prism 6 (Graph Pad software Inc., San Diego, CA, USA), previous to the evaluation of the requirements of normality and homogeneity of variance. Significant differences were estimated over three independent replicates at a 95% confidence interval.

Results

Detection of cell viability with increasing concentrations of BaP in *U. lactuca*

The marine alga *U. lactuca* was cultivated in artificial seawater without BapP and with increasing concentrations of BaP corresponding to 1, 10, 50, 100 and 250 μM for 7 days and cell viability was analyzed visualizing the chlorophyll autofluorescence in chloroplasts using confocal microscopy. *U. lactuca* cells exposed to 0 to 10 μM of BaP showed normal chlorophyll autofluorescence whereas those incubated with higher concentrations showed a decrease in autofluorescence (Fig. 1). It was not possible to determine if BaP was detrimental for its growth and development since the alga has a slow growth rate in vitro. Thus, the sub-lethal concentration of 5 μM of BaP was chosen for further experiments.

BaP is rapidly incorporated and metabolized in *U. lactuca*

To determine whether BaP is incorporated and metabolized in *U. lactuca*, the alga was cultivated in seawater with 5 μM (1.7 μmol in 300 mL) of BaP for 0–72 h and the level BaP was determined in seawater and in the alga. The level of BaP in seawater rapidly decreased until 6 h reaching 0.2 μmol in 300 mL and reached a minimal level at 24 h of 0.04 μmol in the culture medium. The levels of BaP in culture medium without the alga presented no significant changes (Fig. 2a). In contrast, the level of intracellular BaP increased until 6 h reaching a level of 0.6 μmol in 10 g FT and decreased to reach 0.35 μmol in 10 g FT of alga at 72 h (Fig. 2b). Thus, BaP is rapidly removed from the culture medium, reaching 64% of biotransformation at 6 h, and 79% at 72 h in *U. lactuca*.

BaP induced oxidative stress in *U. lactuca*

To determine whether BaP induced oxidative stress in *U. lactuca*, the alga was cultivated in seawater without BaP addition (control) and with 5 Mμ of BaP for 0 to 72 h, and the levels of hydrogen peroxide, superoxide anions and lipoperoxides were determined. The level of hydrogen peroxide initially was 0.1 nmol g^{-1} of FT and it increased to 1 nmol g^{-1} of fresh tissue (FT) at 0.5 h, increased again to 1.3 nmol g^{-1} of FT at 3 h, and again to 1.1 nmol g^{-1} of FT at 6 h, and slowly decreased to 0.35 nmol g^{-1} of FT until 12 h, and remained at this level until 72 h (Fig. 3a). The level of superoxide anions initially was 2.9 nmol g^{-1} of FT, increased to 8.1 nmol g^{-1} of FT at 4 h, to 9.9 nmol g^{-1} of FT at 10 h, to 11.3 nmol g^{-1} of FT at 24 h and decreased to 6.8 nmol g^{-1} of FT at 72 h (Fig. 3b). The level of lipoperoxides initially
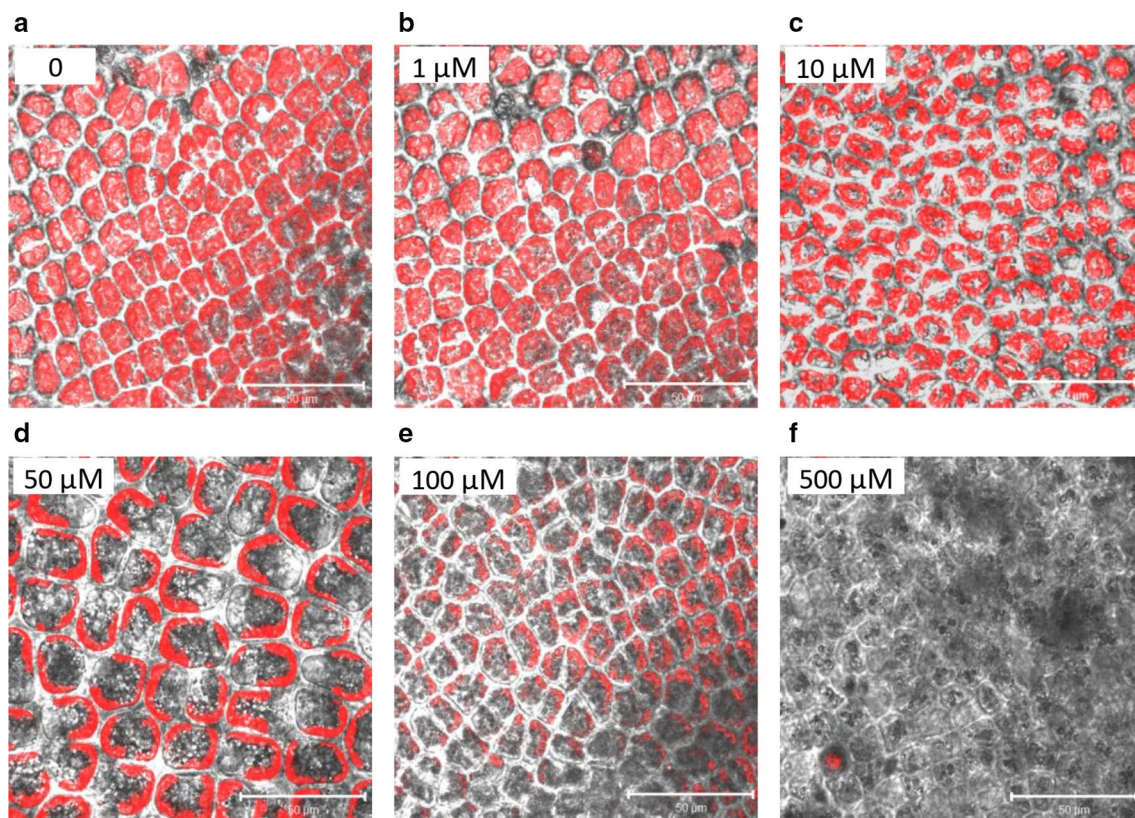
Fig. 1 Visualization of *U. lactuca* cells cultivated with 0–250 μM of benzo[*a*]pyrene for 7 days. The viability of the cells was analyzed based on red autofluorescence of chlorophyll in chloroplasts by confocal microscopy

was 0.4 nmol g^{-1} of FT, it increased to 0.8 nmol g^{-1} of FT at 6 h and slowly decreased until 72 h reaching 0.6 nmol g^{-1} of FT (Fig. 3c). Thus, BaP induced oxidative stress since it increased the levels of hydrogen peroxide, superoxide anions and lipoperoxides in *U. lactuca*.

BaP induced activation of antioxidant enzymes in *U. lactuca*

To analyze whether the increase in oxidative stress induced the activation of antioxidant enzymes, the alga was cultivated in seawater without BaP addition (control) and with 5 μM of BaP for 0 to 72 h, and the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (AP), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and glutathione peroxidase (GP) were analyzed. The activity of SOD was initially 0.4 μmol min^{-1} mg^{-1} of protein, it increased to 1.5 μmol min^{-1} mg^{-1} of protein at 12 h, decreased to 0.8 μmol min^{-1} mg^{-1} of protein at 24 h and slightly increased to 1 μmol min^{-1} mg^{-1} of protein at 72 h (Fig. 4a). The activity of CAT was initially 13.9 μmol min^{-1} mg^{-1} of protein, it increased to 29.5 at 3 h, decreased to 24 μmol min^{-1} mg^{-1} of protein at 6 h, remained stable until 24 h and increased again until 72 h reaching

46 μmol min^{-1} mg^{-1} of protein (Fig. 4b). The activity of AP was initially 87 μmol min^{-1} mg^{-1} of protein, it increased to 264 μmol min^{-1} mg^{-1} of protein at 12 h and decreased to 147 μmol min^{-1} mg^{-1} of protein at 72 h (Fig. 4c). In contrast, the activity of DHAR remained unchanged until 72 h showing activity of around 20 μmol min^{-1} mg^{-1} of protein (Fig. 4d). The activity of GR was initially 110 μmol min^{-1} mg^{-1} of protein, it increased to 254 μmol min^{-1} mg^{-1} of protein at 3 h, decreased to 160 μmol min^{-1} mg^{-1} of protein at 24 h and slightly increased to 211 μmol min^{-1} mg^{-1} of protein at 72 h (Fig. 4e). The activity of GP was initially 29 μmol min^{-1} mg^{-1} of protein, it increased to 61 μmol min^{-1} mg^{-1} of protein at 6 h, decreased to 39 μmol min^{-1} mg^{-1} of protein at 24 h and slightly increased to 53 μmol min^{-1} mg^{-1} of protein at 72 h (Fig. 4f). Thus, most of the antioxidant enzyme activities showed a maximal activity from 3–12 h, except CAT that showed it maximal activity at 48–72 h and DHAR remained unchanged.

BaP increased the level transcripts encoding antioxidant enzymes in *U. lactuca*

To analyze whether the increase in activities of antioxidant enzymes is due to an enhanced their expression,
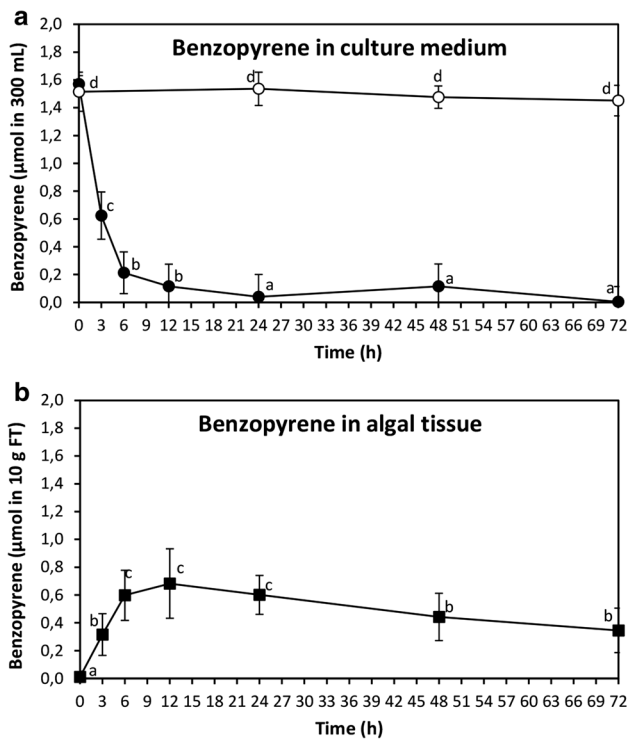
Fig. 2 Level of benzopyrene (BaP) in the culture medium (**a**) and in the marine alga *U. lactuca* (**b**) cultivated with 1.7 μmol in 300 mL (5 μM) of benzopyrene (BaP) for 0–72 h. Black circles represent the culture with the alga, open circles represent the culture without the alga. Symbols represent mean values of three independent experiments ± SD. Letters indicate significant differences among experiments ($P < 0.05$)

the alga was cultivated in seawater without BaP addition (control) and with 5 Mμ of BaP for 0–72 h and the relative level of transcripts encoding SOD, CAT, AP, DHAR, GR and GP were determined. The relative level of transcripts encoding SOD increased showing a maximal level of 0.4 times of increase at 24 h (Fig. 5a). The relative level of transcripts encoding CAT increased showing a maximal level of 1 time of increase at 48 h (Fig. 5b). The relative level of transcripts encoding AP increased showing a maximal level of 0.6 times of increase at 12 h (Fig. 5c). The relative level of transcripts encoding DHAR remained unchanged until 72 h (Fig. 5d). The relative level of transcripts encoding GR increased showing a maximal level of 9 times of increase at 12 h (Fig. 5e). The relative el of transcripts encoding GP increased showing a maximal level of 1.7 times of increase at 12 h (Fig. 5f). Thus, the increase in the level of transcripts encoding antioxidant enzymes showed maximal levels at 12–48 h, except for DHAR which remained unchanged.
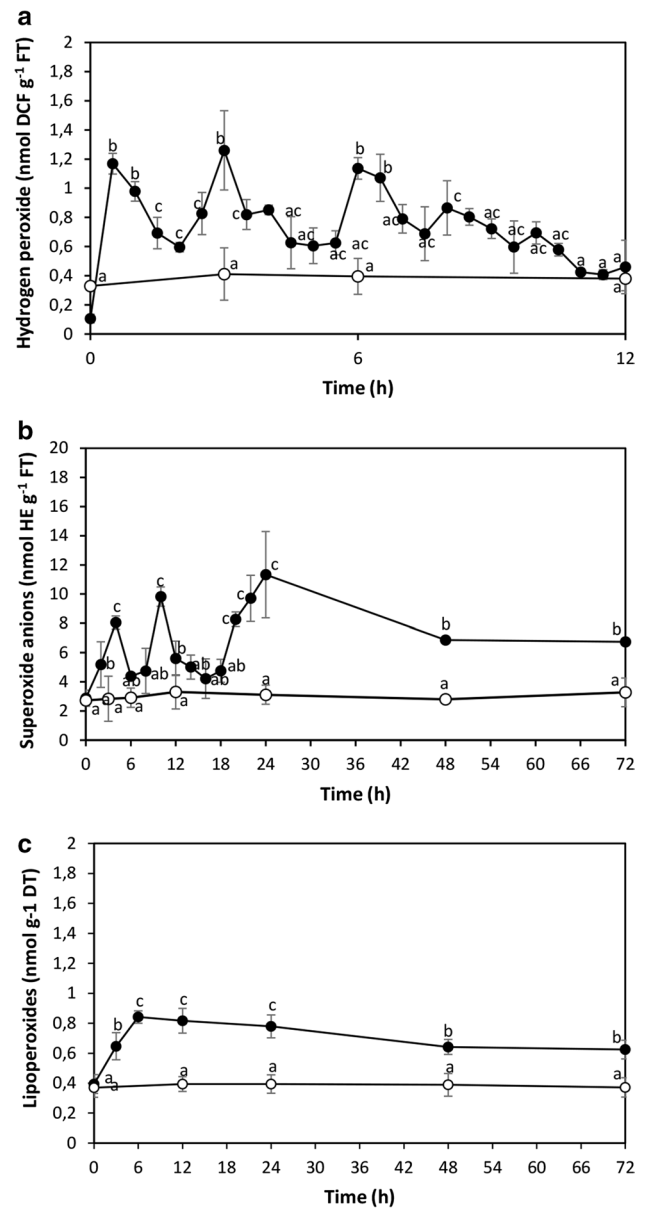

Fig. 3 Level of hydrogen peroxide (**a**), superoxide anions (**b**) and lipoperoxides in *U. lactuca* cultivated without benzopyrene (BaP) addition (open circles) and with 5 μM of BaP for 0–72 h (black circles). The level of hydrogen peroxide, superoxide anions and lipoperoxides are expressed as nanomoles per gram of fresh tissue (FT). Symbols represent mean values of three independent experiments ± SD. Letters indicate significant differences among experiments ($P < 0.05$)

BaP increased CYP450 activity and GST transcripts and activity in *U. lactuca*

To analyze the mechanisms involved in BaP detoxification, the alga was cultivated in seawater without BaP addition (control) and with 5 Mμ of BaP for 0 to 72 h, and the activity and the relative level of transcripts of
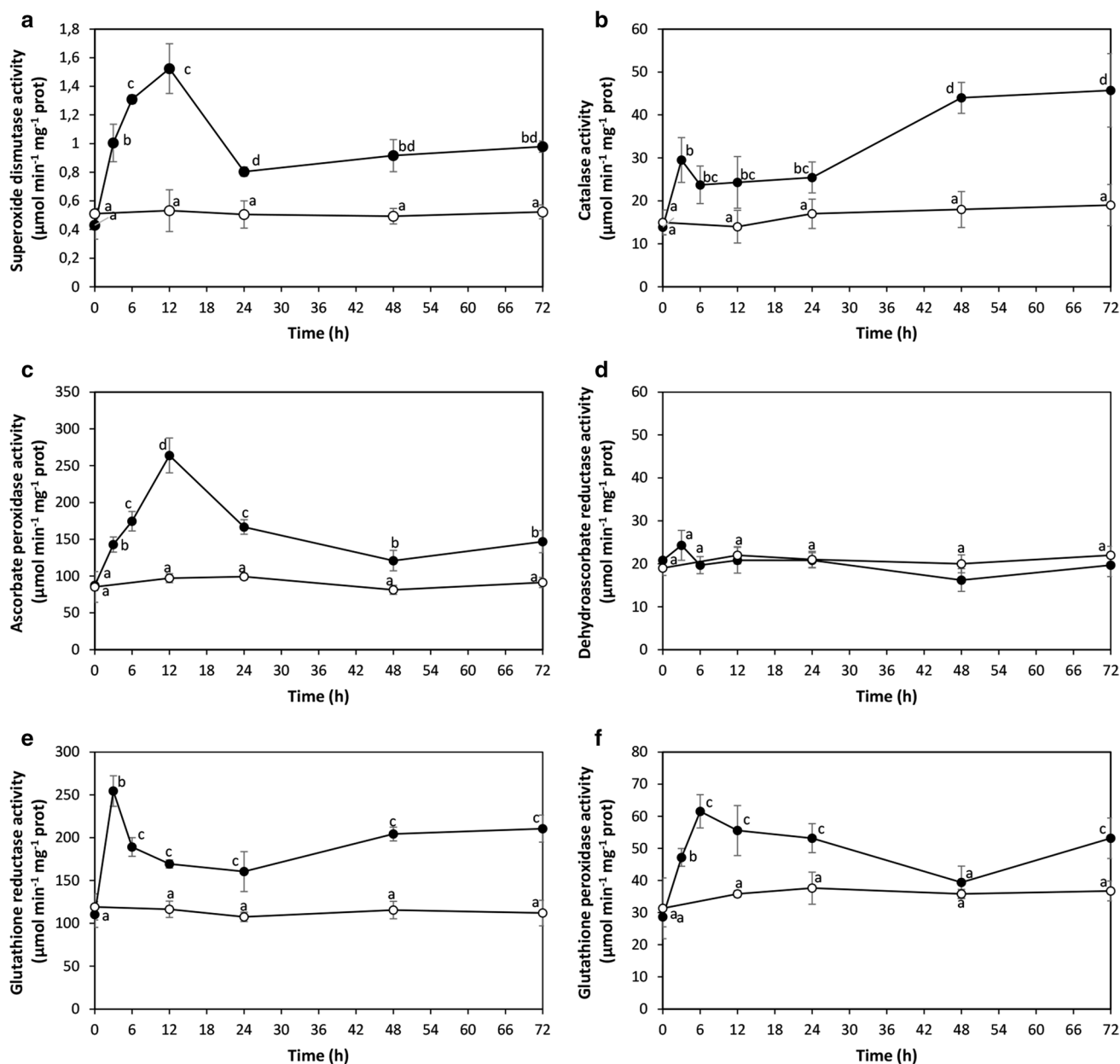
Fig. 4 Activities of antioxidant enzymes superoxide dismutase (**a**), catalase (**b**), ascorbate peroxidase (**c**), dehydroascorbate reductase (**d**), glutathione reductase (**e**) and glutathione peroxidase (**f**) in *U. lactuca* cultivated in seawater without benzopyrene (BaP) addition (open circles) and with 5 μM BaP for 0–72 h (black circles). The activities of antioxidant enzymes are expressed as micromoles per minute per milligram of fresh tissue (FT). Symbols represent mean values of three independent experiments ± SD. Letters indicate significant differences among experiments ($P < 0.05$)

glutathione-S-transferase (GST) were analyzed. The activity of GST was initially 18 μmol min^{-1} mg^{-1} of protein and it increased showing a maximal level at 6 and 12 h of 68 and 63 μmol min^{-1} mg^{-1} of protein, respectively, and decreased to control level at 72 h (Fig. 6a). The relative level of transcripts encoding GST increased showing a maximal level of five times at 3 h and then decreased to control level (Fig. 6b).

Thus, the activity of GST and the level of transcripts encoding GST increased in response to BaP.

In addition, the alga was cultivated without BaP (control), with 5 Mμ (1,7 μmol in 300 mL) of BaP, with an inhibitor of CYP450, Cu^{+2} ions, and with 5 Mμ of BaP, and with an inhibitor of GSH synthesis, buthionine sulfoximine (BSO), and with 5 Mμ BaP, and the level of BaP was analyzed in algal tissue. The level of BaP in the alga cultivated with Cu^{+2} and
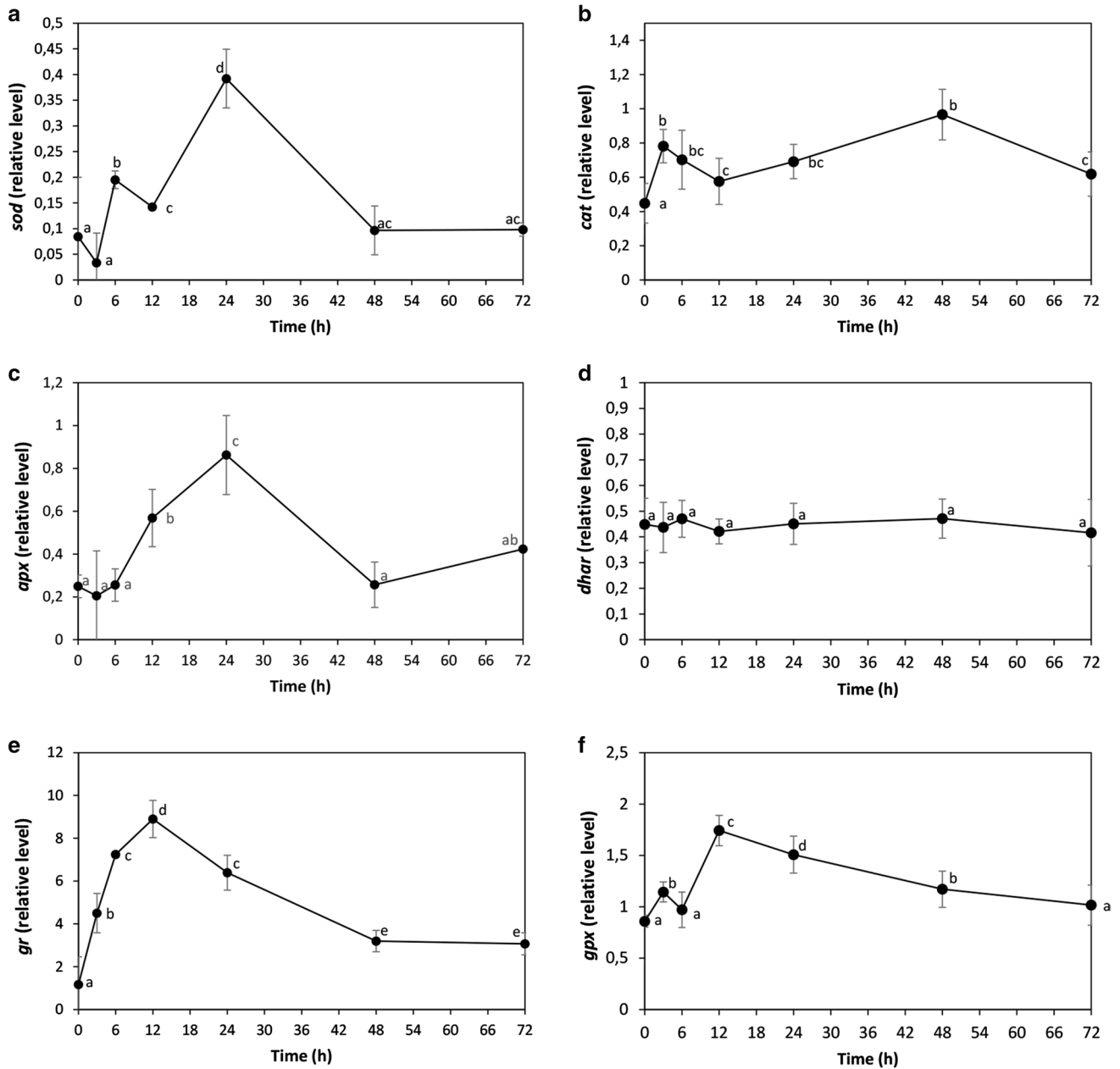
Fig. 5 Level of transcripts encoding antioxidant enzymes superoxide dismutase (**a**), catalase (**b**), ascorbate peroxidase (**c**), dehydroascorbate reductase (**d**), glutathione reductase (**e**) and glutathione peroxidase (**f**) in *U. lactuca* cultivated in seawater without benzopyrene (BaP) addition (open circles) and with 5 μM BaP for 0–72 h (black circles). The relative level of transcripts is expressed as $2^{-\Delta\Delta CT}$. Symbols represent mean values of three independent experiments $\pm$ SD. Letters indicate significant differences among experiments ($P < 0.05$)

BaP slightly decreased at 12 h of culture in 17% compared to control but increased at 24 h of culture in 81% compared to control (Fig. 6c). The level of BaP in the alga cultivated with BSO and BaP increased at 12 h of culture in 32% and at 24 h of culture in 68% compared to control (Fig. 6d). Thus, a CYP450 and GST are involved in detoxification of BaP in *U. lactuca*.
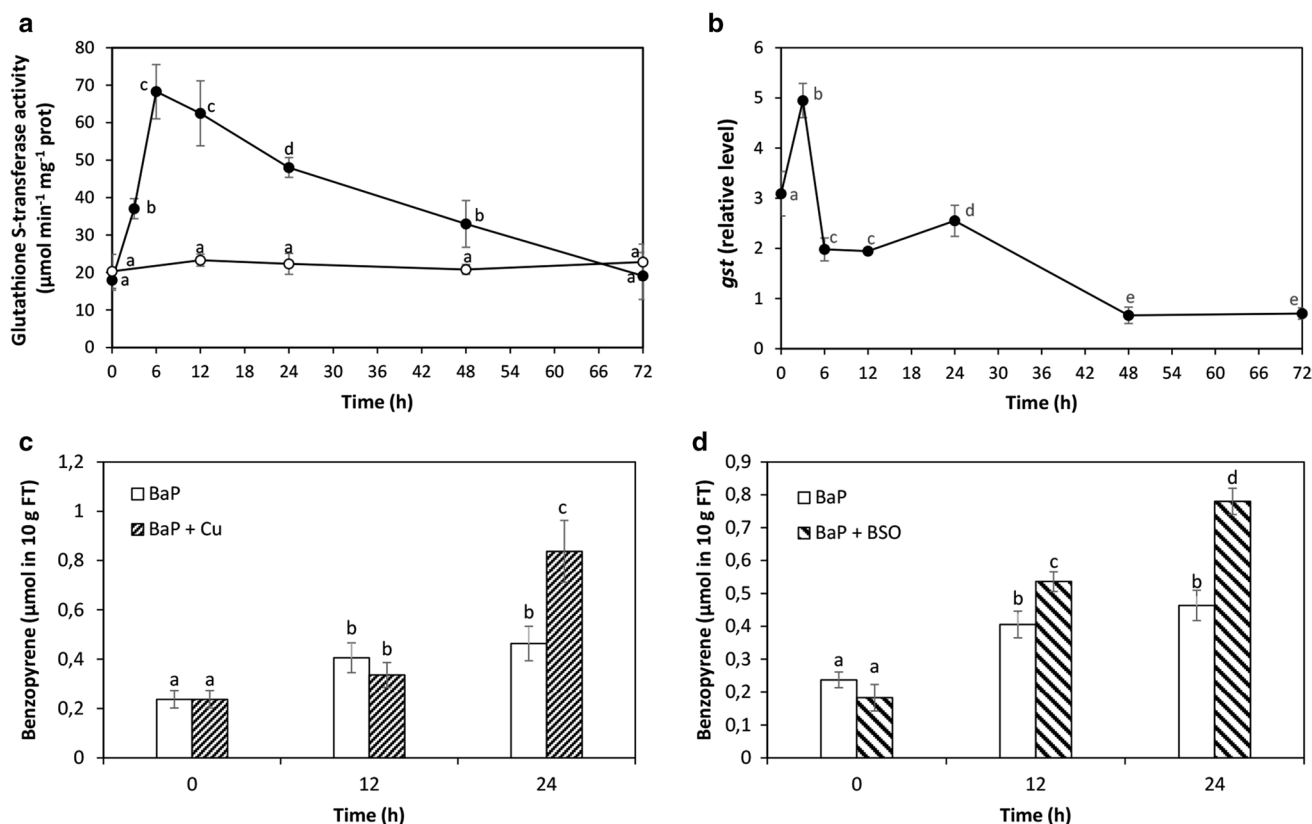
Fig. 6 Activity of the detoxification enzyme glutathione-S-transferase (**a**) and relative level of transcripts encoding GST (**b**) in *U. lactuca* cultivated in seawater without benzopyrene (BaP) addition (open circles) and with 5 μM BaP for 0–72 h (black circles). The activity is expressed as micromoles per milligram of fresh tissue (FT) and the relative level of transcripts as $2^{-\Delta\Delta CT}$. Symbols represent mean values of three independent experiments ± SD. Level of BaP in the alga cultivated without BaP addition for 0, 12 and 24 h (open bar, **c** and **d**), with 1 mM Cu^{+2} and 5 μM BaP (hashed bar, **c**) or with 0.5 mM buthionine sulfoximine (BSO) 5 μM BaP (hashed bars, **d**) for 0, 12 and 24 h. Bars represent mean values of three independent experiments ± SD. Letters indicate significant differences among experiments ($P < 0.05$)

Discussion

In this work, it was shown that BaP is rapidly absorbed in *U. lactuca* reaching a biotransformation of 64% at 6 h, and 79% at 12 h, and that photodegradation or natural decaying of the compound did not occur in the culture medium. These results showed that *U. lactuca* is more efficient than other green macroalgae to degrade BaP than *U. intestinalis* and *C. glomerata* that metabolized less than 50% of BaP at 6 h of culture (Kirso and Ihra 1998). In contrast, detoxification of PAHs in brown macroalgae appeared to be less efficient since *F. vesiculosus* and *C. filum* metabolized only 4% after at 5 days and the brown macroalga *L. japonica* cultivated with 0.5 μM of PHE and PYR showed complete biotransformation of these PAHs after 14 days (Wang and Zhao 2007). In addition, it has been determined that *U. lactuca* can also accumulate and metabolize ANT (A. González, unpublished data) indicating that other PAHs may also be removed from seawater by *U. lactuca*.

On the other hand, it was shown that BaP induce an oxidative stress condition in *U. lactuca* since hydrogen peroxide, and mainly superoxide anions, accumulate in the alga. Similar results have been obtained in *A. thaliana* cultivated with 1.25 mM PHE for 30 days showing an increase in hydrogen peroxide and lipoperoxides which indicate that PHE induced an oxidative stress condition in plants (Liu et al. 2009). In addition, the aquatic liverwort *R. fluitans* cultivated with 10 μM of PHE for 72 h showed an increase in hydrogen peroxide, lipoperoxides and carbonylated proteins indicating that PHE induced an oxidative stress condition (Burritt 2008). Interestingly, the level of lipoperoxides in *U. lactuca* showed a maximal level of only 0.8 nmol g^{-1} of FT, which is just a small fraction of the 15 nmol g^{-1} of FT observed in *R. communis* cultivated with 0.5 μM of PHE (Burrit 2008). Thus, *U. lactuca* may possess a highly efficient mechanism to mitigate oxidative stress. In addition, *U. lactuca* presents a higher threshold of tolerance to BaP, up to 50 μM for

7 days without alteration in cell morphology indicating that *U. lactuca* may be a good candidate for remediation of PAHs from seawater.

The oxidative stress triggered by BaP induced the increase in activities of antioxidant enzymes SOD, CAT, AP, GR and GP in *U. lactuca*. Interestingly, the transcriptional activation occurred after the increases in activities of antioxidant enzymes, which indicates the enhanced enzyme activities at initial times are not transcriptionally regulated. This additive response of antioxidant enzymes may explain the efficient buffering of hydrogen peroxide and the increase in superoxide anions that began at 24 h and remained until 72 h. Interestingly, GR and GP showed the highest increases in activities and in the level of transcripts compared with SOD, CAT and AP, indicating that these enzymes are the principal antioxidant enzymes that mitigate BaP-induced oxidative stress. It is important to mention that transcripts encoding GR and GP and GST enzymes that uses GSH as substrate were increased. In this sense, the mussel *Perna viridis* cultivated in seawater contaminated with PAHs for 30 d showed the activation of antioxidant and metabolizing enzymes such GR, GP and GST as well as the synthesis of GSH and the parameter that better correlated with PAHs accumulation in the tissue was GSH (Cheung et al. 2001). Interestingly, DHAR activity that recycle dehydroascorbate to ascorbate with consumption of GSH did not show an increase indicating that the Haliwell-Asada-Foyer cycle, involving AP, DHAR and GR is uncoupled (Foyer and Noctor 2011). A possible explanation for the latter is that GSH is synthesized in response to BaP but it is quickly channeled as a substrate for GR, GP and GST activities but the latter need to be further analyzed. These results support the idea that GSH is an important molecule for PAHs metabolism in animals and marine algae.

Furthermore, it was shown that the level of transcripts and the activity of GST were increased in response to BaP in *U. lactuca*. The increase in transcripts occurred before the increase in activity indicating that the increase in GST activity is transcriptionally regulated. This is in accord with the findings in maize plants cultivated in petroleum-contaminated soil showed an increase in the level of transcripts encoding five different GSTs (Cever-Keshin et al. 2018). In addition, it was shown that inhibitors of CYP450 and GST activities increased the level of BaP in algal tissue suggesting that these enzymes are involved in BaP biotransformation in *U. lactuca* (Fig. 7). The increase in CYP450 activity suggests that dihydrodiols derivatives of BaP may be synthesized in *U. lactuca*. In this sense, it has been shown in the green microalgae *S. acutus* and *A. braunii* cultivated with 1200 mg L^{-1} BaP for 48 h almost completely metabolized BaP and accumulate dihydrodiol-derivatives of this compound (Warshawski et al. 1995). In addition, the green microalga *S. capricornutum* cultivated with 266 ng L^{-1} for
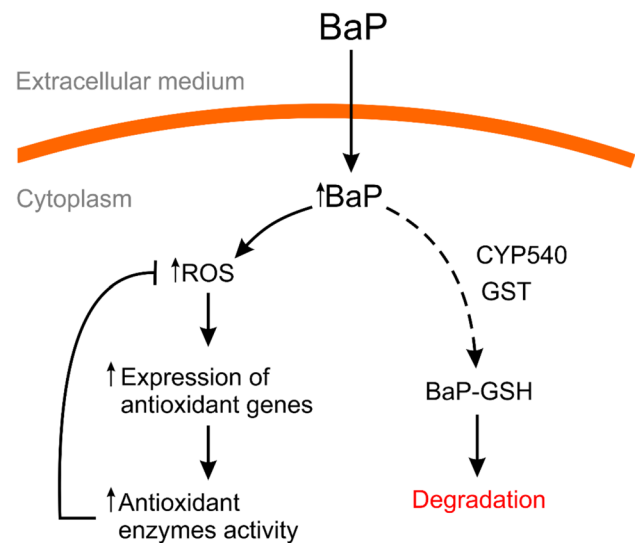

Fig. 7 Model of BaP detoxification in the marine alga *Ulva lactuca*. The alga exposed to BaP rapidly absorbs the xenobiotic and showed an increase BaP at the intracellular level. The accumulation of BaP induces oxidative stress which may activate the expression of antioxidant enzyme genes increasing their activities to mitigate oxidative stress and cell damage. On the other hand, intracellular BaP is the substrate for enzymes such as CYP540 and GST that allowed its oxidation and further conjugation with GSH leading to its degradation

48 h can also metabolize BaP to dihydrodiol derivatives, mainly 4,5 dBaP, 7,8 dBaP and 9,10 dBaP (Hernández-Blanco et al. 2016). Furthermore, the green microalga *S. capricornutum* cultivated 1160 µg L^{-1} of BaP for 4 days metabolize BaP to 4,5 dBaP dihydrodiol and further conjugate these molecules with glucose or sulphate (Warshawski et al. 1990). The conjugation of the oxidized PAHs with water-soluble molecules allows their translocation, accumulation, and transformation (Hernandez-Vega 2017). In this sense, it has been shown that *A. thaliana* cultivated with PHE showed an increase in the level of transcripts encoding several isoforms of GST that may allow the accumulation the PHE-GSH moieties in the vacuole (Weisman et al. 2010). Thus, it is possible that dihydrodiols derivatives may be synthesized from BaP that are further conjugated with GSH to allow its degradation in *U. lactuca,* but the latter need to be further analyzed.

Conclusions

The marine alga *Ulva lactuca* cultivated with BaP displayed a rapid accumulation and metabolism of this PAH. BaP induced oxidative stress due to the accumulation of hydrogen peroxide and superoxide anions and oxidative stress produced lipoperoxides from membrane fatty acids. To buffer oxidative stress, the activities of antioxidant enzymes SOD,

AP, GR and GP were enhanced, and these increases are, at least in part, transcriptionally regulated. The activation of CYP450 and GST suggests that the alga metabolize BaP for its detoxification.

Author contribution statement AG performed the alga cultures, confocal microscopy, BaP extractions, HPLC analysis, ROS determinations, enzyme activities assays, data analysis and prepared the figures, DE performed BaP extractions, cultures with inhibitors, and qPCR assays, CV performed in vitro cultures and HPLC analysis. AM supervised of workflow and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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