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Single and Combined Effects of Cypermethrin and UVR Pre-Exposure in the Microalgae *Phaeodactylum Tricornutum*

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Abstract

Coastal marine microalgae are exposed to anthropogenic pollutants, including pesticides from aquaculture/agriculture/household uses. Some microalgae species, such as *Phaeodactylum tricornutum*, can induce and accumulate UV-absorbing compounds (UACs) upon ultraviolet radiation (UVR) exposure to prevent deleterious effects. Tolerance mechanisms activated by natural stressors might also protect organisms from anthropogenic stressors. This work assesses the effects of the insecticide cypermethrin (Cyp) and UVR in the marine microalgae *P tricornutum.* Considering the pro-oxidant properties of both stressors and UACs' induction in *P tricornutum*, lethal and sublethal effects of Cyp were tested in cultures with and without UVR acclimation. After a 24-h exposure to 10 μg L^{-1} of technical Cyp or culture medium, UACs, growth, glutathione-S-transferase activity (GST), sulfhydryl groups (SH-g), and lipid peroxidation (LPO) were analyzed. Results showed differences in terms of growth between Cyp and Cyp + UVR pre-exposure. UACs' content was induced after UVR acclimation and diminished after 24 h of growth in control and UVR pre-treated cultures, while levels remained constant under Cyp exposure. A single Cyp exposure exerted GST induction, SH-g depletion, and LPO increments. In UVR-acclimatized treatments, oxidative stress responses showed similar or more pronounced effects than the single chemical exposure, suggesting a potential additive effect of the UVR acclimation. The contrasting effects of Cyp + UVR observed between growth and biochemical responses suggest different compensatory mechanisms that need to be further investigated. Also, it highlights the need to include both lethal and sublethal endpoints to understand microalgae's tolerance and its significance in the multiple stressors' context.

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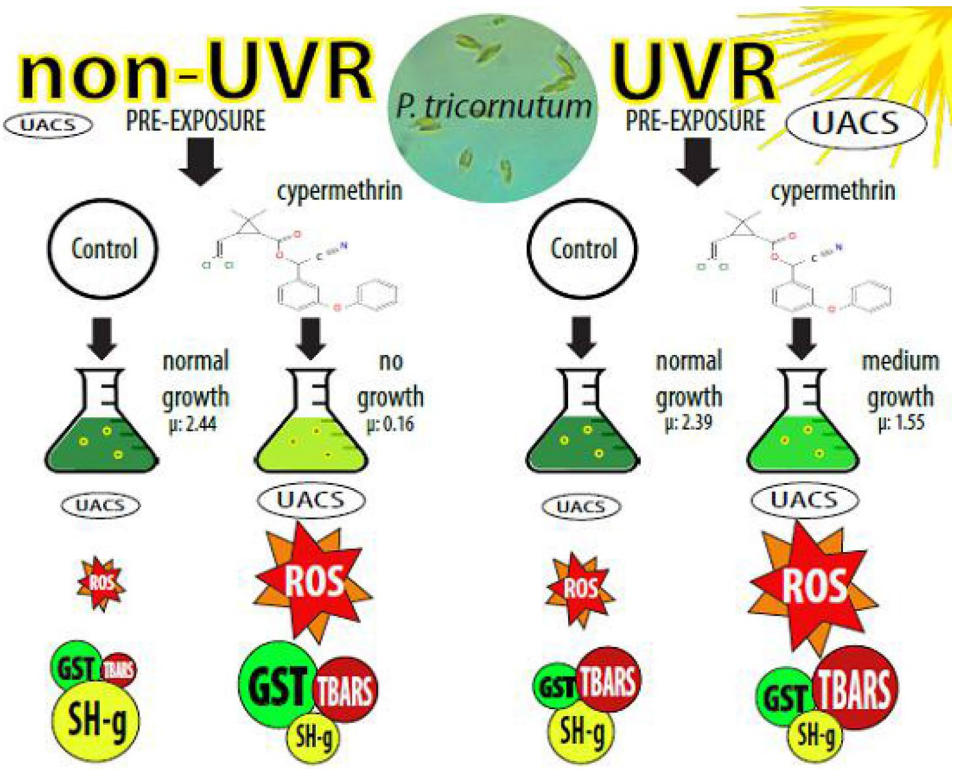
Graphical Abstract



The biological impact of multiple stressors is one of the most significant concerns in complex environments such as coastal areas (Vinebrooke et al. 2004; Pelletier et al. 2006). Natural fluctuation of environmental factors such as temperature, salinity, radiation, or predation might also modify the effect of chemical stressors from anthropogenic activities (Fischer et al. 2013). In this scenario, it is essential to consider the relationship between the acquired tolerance of organisms to natural stressors and their sensitivity toward other stressors, such as synthetic chemicals (Pelletier et al. 2006; Fischer et al. 2013). The type of response would depend on the organization level and the modes of action of both the pollutant and the environmental stressor (Vinebrooke et al. 2004; Fischer et al. 2013). Then, a tolerance mechanism activated by the exposure to natural stressors might also protect the organism against the effect of other stressors, and a co-tolerance scenario is proposed (Fischer et al. 2010, 2013). Solar ultraviolet radiation (UVR, 280–400 nm) represents one of the most important natural stressors for phytoplankton organisms. Its action reduces growth and photosynthesis rates and increases reactive oxygen species (ROS) content, producing deleterious effects at different cellular levels (Babu et al. 2003; Helbling et al. 2003; Hernández Moresino and Helbling 2010). Mechanisms that prevent damage from UVR are expected to act against other stressors that also generate ROS formation (Korkaric et al. 2015). Marine microalgae represent non-target aquatic organisms that are commonly exposed to anthropogenic pollutants, including pesticides. They also show higher sensitivity to pollution than freshwater species (Lozano et al. 2014). Induction and accumulation of UV-absorbing compounds (UACs, such as mycosporine-like amino acids among others), flavonoids, or carotenoids can help some microalgae/macroalgae species to cope with the pro-oxidant UVR effects (Marcoval et al. 2007; Navarro et al. 2008), while chemical stress can also produce the induction of both enzymatic and non-enzymatic antioxidant responses (Bhargava et al. 2007; Wang et al. 2011). Synthetic pesticides used in agriculture could reach aquatic environments near or far from sources such as estuarine and coastal waters (Munaron et al. 2012; Silva-Barni et al. 2019). In this sense, most of the studies on the tolerance of microalgae to pesticides are focused on herbicides due to their action on the photosynthetic system (Jamers and De Coen 2010; Lozano et al. 2014) and mainly on freshwater species (Nestler et al. 2012; Baruah and Chaurasia

2020). However, information about the effect of insecticides or fungicides, designed to act against non-photosynthetic organisms, in coastal marine microalgae is scarce (Wang et al. 2011; Dupraz et al. 2019). Particularly, some pesticides, such as the pyrethroid insecticide cypermethrin (Cyp), could be used in agricultural fields and aquaculture activities or for domestic pest control (Burridge et al. 2000; Tucca et al. 2014; Gao et al. 2016). Once in the aquatic system, due to its high hydrophobicity (Cyp log Kow = 6.3), Cyp is rapidly adsorbed to suspended particulate matter and colloidal organic matter, reaching bottom sediments and adsorbed to plankton community (Yang et al. 2006; Fojut and Young 2011). Although little data about Cyp occurrence in coastal areas are available, environmental detected levels in the water column vary with a broad range of values from the μg L^{-1} in areas near commercial aquaculture to the ng L^{-1} scale (Willis et al. 2005; Feo et al. 2010; Bhattacharjee et al. 2012). Thus, when used to control sea lice infestation, the recommended dose is 5 μg L^{-1} (Willis and Ling 2004), and Cyp values around fish culture area were reported in the order of 0.11–0.218 μg L^{-1} (Willis et al. 2005; Wang et al. 2012). These variable levels of Cyp could be linked with different environmental scenarios and could lead to differences in their toxic effect for aquatic organisms from sublethal to lethal effects (Friberg-Jensen et al. 2003; Tucca et al. 2014). Growth inhibition and the induction of oxidative stress were reported at 50 μg L^{-1} for 96 h in several marine microalgae (Wang et al. 2011), while for the freshwater microalgae *Chlorella sp.*, the same effects were observed in the range of 11–48 mg L^{-1} (Baruah and Chaurasia, 2020). These observations support the higher sensitivity of marine microalgae regarding freshwater species. The induction of antioxidants defenses during acclimation to UVR and its role on the tolerance to cadmium, Irgarol 1051, diuron, atrazine, or paraquat was reported for freshwater and marine microalgae (Zhang et al. 2008; Sjollema et al. 2014; Korkaric et al. 2015). However, studies related to the influence of natural stressors and pesticides in marine microalgae are scarce (Coquillé et al. 2018). Besides, to the best of our knowledge, there is no information on the sublethal effect after combined exposure to cypermethrin and UVR-acclimatized cultures in marine microalgae species.

Phaeodactylum tricornutum is a widely distributed marine microalgae, frequently used in aquaculture and recommended for microalgal toxicity testing due to their ease of culture, lack of toxicity, and high nutritional value (Moreira et al. 2006). Besides, it is known to produce substances with antioxidant properties under UVR exposure (Jeffrey et al. 1999). Since interactions of chemical mixtures and their effects are relatively addressed (Fischer et al. 2013), the study of anthropogenic/natural stressors mixtures and acclimation influence emerges as a complex topic to be addressed to assess the effects of currently used pesticides in more realist scenarios. This work aimed to evaluate the following hypothesis: (i) cypermethrin exposure induces deleterious effects in *P. tricornutum,* including oxidative stress, and (ii) the acclimation of *P. tricornutum* to UVR exposure induces mechanisms of tolerance that might help the microalgae to cope with the pro-oxidant effects of cypermethrin.

Materials and Methods

Microalgae Culture

The marine microalgae *P. tricornutum* was cultured in F/2 Guillard medium (Guillard, 1973), in 0.22-μ filtered seawater (FSW; S = 33), and kept in 6-L glass containers under semi-continuous cultures. The cultures had constant aeration and were exposed to photosynthetically active radiation (PAR) of 65.4 W m^2 (fluorescent tubes 40 W) at 22 °C. Additionally, to photoinduce UACs, the cultures were inoculated into F/2 medium in 300-mL polycarbonate containers transparent to UVR and exposed for 20 days to artificial lamps (Q-Panel UVA-340 for UVR and Philips daylight for PAR) under irradiance of 65.4 W m^{-2} for PAR and 20 W m^{-2} for UVR (320–400 nm, Marcoval et al. 2007). The spectral absorption characteristics of *P. tricornutum,* before and after 20 days exposition to PAR and PAR + UVR, are shown in Fig. 1S. UACs' content was analyzed from 50 ml of culture subsample (T_0) filtered onto a Whatman GF/F glass fiber filter (25 mm). UACs were extracted from filters overnight in absolute methanol (Hernández-Moresino and Helbling 2010), and samples were centrifuged for 15 min at 800 g. The UACs' content was estimated by peak heights at 330 nm of the spectra scans between 280–750 nm via a scanning spectrophotometer (Shimadzu UV-2102 PC, UV/Visible) and expressed as optical density (OD).

Experimental Design

EC50 Calculation

A 96-h EPA toxicity test for microalgae was performed (USEPA 2002). Cultures in exponential phase were inoculated in 500-mL Erlenmeyer flask containing 350 ml of culture medium with crescent concentrations of technical cypermethrin (Gleba 25% w/v): 0 , 20 , 100 , 200, 500 μg L^{-1}, selected based on Wang et al. (2011, 2012). All solutions were prepared from a stock solution of 250 mg L^{-1}. Control performed with F/2 Guillard medium (without microalgae) was established to evaluate the physicochemical modification of the insecticide. Erlenmeyers were manually vortex to avoid cell sedimentation. The assay was run by triplicate under PAR of 65.4 W m^{-2} (fluorescent tubes 40 W) at a temperature of 20 °C (± 2 °C). Growth was measured at

0, 12, 24, 48, and 96 h (Fig. 2S), and EC_{50} was calculated using the free software TRAP versión 1.30a USEPA, 2015 (Fig. 3S), Table 1.S).

Sublethal Toxicity Test (24 h)

Exposure concentration of 10 µg L^{-1} of technical Cyp was selected considering the EC_{50} value of 81 µg L^{-1} calculated from the 96-h test and the usage dose in the coastal marine environment at 5 µg L^{-1}. Microalgae from cultures with and without UVR treatment, in exponential phase, were inoculated in 500-mL Erlenmeyer containing 350 mL of culture medium with and without a final concentration of 10 µg L^{-1} of technical Cyp (Gleba 25% w/v). All cultures were kept for 24 h under PAR of 65.4 W m^{-2}(fluorescent tubes 40 W) at 20 °C (± 2 °C). The exposure treatments were named as: a) control: non-UVR, non-Cyp, b) UVR: UVR pre-exposure, non-Cyp, c) Cyp: non-UVR, + Cyp, and d) UVR-Cyp: UVR pre-exposure + Cyp. Each treatment was run in triplicate.

Culture growth was monitored, taking 3 ml of cell cultures at the beginning (T_0) and after 12 (T_{12}) and 24 (T_{24}) h. Aliquots were immediately fixed with buffered formaldehyde (final concentration in the sample = 0.4% v/v of formaldehyde), colored by adding a drop of rose bengal, and kept in the dark at room temperature. The cell count was performed in a Neubauer chamber under a light microscope with 400 X magnification. Specific growth rate (µ) was calculated according to Guillard (1973) modified by Guillard and Sieracki (1973) as $\mu = (3.322 \div \Delta t) \times (\log N2 \div N1)w$ here Nx is the culture density at time x and Δt is the delta on final and initial time. After 12 (T_{12}) and 24 (T_{24}) h, subsamples of 50 mL were taken from each treatment to analyze UACs' content as described in *Organisms and maintenance media,* while subsamples of 350 mL were used for sub-individual responses.

Cypermethrin Levels in Culture Media

Levels of Cyp were evaluated to know its persistence under the experimental conditions. Thus, 200 mL of F/2 Guillard medium spiked with 50, 250, and 500 µg L^{-1} of technical Cyp was incubated in triplicate, with and without microalgae inoculums. Subsamples of 5 mL were taken at the beginning (T_0) and after 24 h (T_{24}), centrifuged at 3000 g and 4 °C for 10 min to eliminate cells and any debris. Cyp was analyzed by liquid–liquid extraction by shaking end-over-end for 2 h with a mixture of "dichloromethane:hexane (2:1, v/v)." All samples were handled on glass or aluminum material prewashed with hexane. Polychlorinated biphenyl (PCB) #103 was used as an internal standard. Cyp was identified and quantified using a Shimadzu GC-ECD 17A equipped with a fused-silica capillary column of 30 m, SPB-5 (0.25 mm i.d., 0.25 µm film thicknesses, Supelco, USA). The oven temperature started at 100 °C, held for 1 min, followed by increases of 15 °C/min up to 180 °C, held for 1 min, then 1.5 °C/min up to 215 °C, and then 15 °C/min up to 290 °C for 13 min. The inject port was set at 275 °C and the detector at 300 °C. The carrier gas was ultrahigh-purity helium (39 mL min^{-1}). Cyp isomers were identified by their relative retention times to #PCB 103. Calibration curves made with technical and pure cypermethrin were run. Detection and quantification limits were 5.92 and 19.73 ng mL^{-1}, respectively. Laboratory and instrumental blanks were included in determining blank corrections by any possible contamination during laboratory handling.

Oxidative Stress Responses

- *Microalgae extract* Cells from 350 mL of culture were concentrated by centrifugation at 3000 g for 10 min at 4 °C. The pellets were resuspended in 1 mL of 0.1 M sodium phosphate buffer (pH 6.5) and then split into two Eppendorf tubes (0.5 mL each) of a known weight and centrifuged again at 3000 g for 10 min at 4 °C. The supernatant was discarded and weighed again to determine pellet weight by difference. The duplicated extracts were immediately frozen in liquid nitrogen and stored at -80 °C for further analysis. Then, the extracts were homogenized with sodium phosphate buffer (0.1 M, pH 6.5) in a 1:2 (w/v) ratio according to Martínez-Domínguez et al. (2008), centrifuged at 15,000 g for 10 min at 4 °C, and the supernatant was stored at -80 °C for further determination of protein content, glutathione-

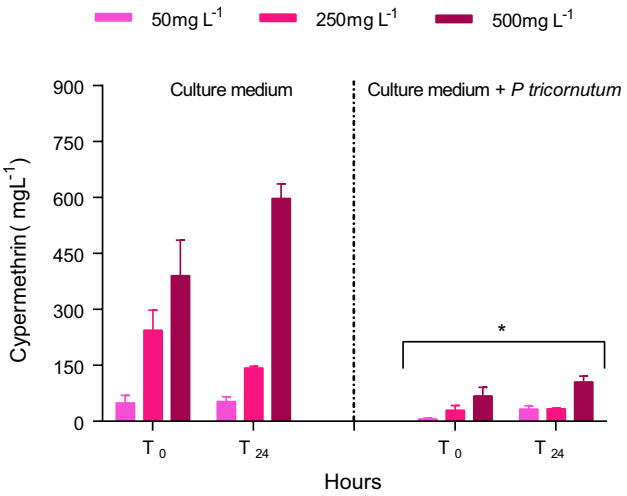


Fig. 1 Technical cypermethrin levels (50, 250, and 500 µg L^{-1}) in culture medium with and without inoculums of *Phaeodactylum tricornutum* after 24 h of growth. Asterisk indicates significant differences between non-inoculated and inoculated medium among all concentrations and for both exposure times

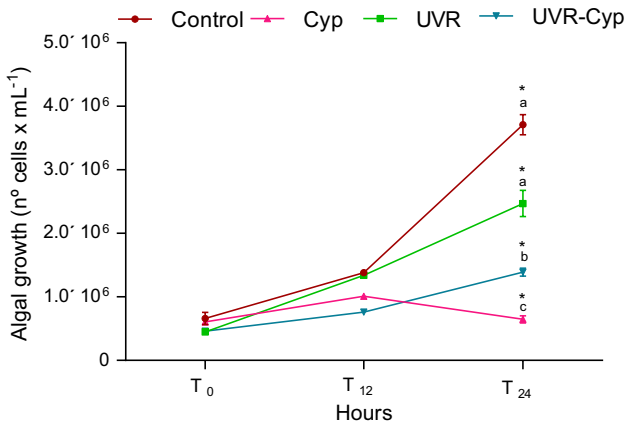


Fig. 2 Microalgae growth in *Phaeodactylum tricornutum* cultures at 0, 12, and 24 h (T_h). Control: microalgae grown in F/2 media; Cyp: microalgae grown in F/2 medium enriched with 10 µg L^{-1} technical cypermethrin; UVR: microalgae pre-exposed to UVR, grown in F/2 medium; UVR-Cyp: microalgae pre-exposed to UVR, grown in F/2 medium enriched with 10 µg L^{-1} technical cypermethrin. Measurements represent the mean ± SE. Different letters indicate significant differences ($p < 0.05$) among all treatments at T_{24}. Asterisk indicates significant differences between time exposure (12, 24 h) within each treatment

S-transferase activity (GST), and sulfhydryl groups (SH-G). Homogenates for lipid peroxidation (LPO) were made from the microalgae extract in a 1:5 ratio with 0.1% (w/v) of trichloroacetic acid (TCA) and used immediately.

- *Protein content* was determined by the Bradford method (1976) using serum albumin as standard and data expressed as µg of proteins × 10^6 $cell^{-1}$.
- *Glutathione-S-transferase activity* GST activity was measured using the methodology described by Habig and Jakoby (1981). This method measures the conjugation at 25 °C of the CNDB (1-chloro-2,4-dinitrobenzene) and reduces glutathione (GSH) to 340 nm. Then, 0.1 M potassium phosphate buffer was used as a reaction medium and using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH in a 1:1 molar ratio. Data were expressed as GST activity: nmol × min^{-1} × mg $protein^{-1}$.
- *Sulfhydryl groups* Total sulfhydryl group (SH-gT), protein sulfhydryl group (SH-gP), and non-protein sulfhydryl group (SH-gNP) were determined according to the methodology of Sedlak and Lindsay (1968). The SH-gT and SH-gNP contents were measured before and after deproteinization of homogenates, respectively, using TCA (50%), and detected using naphthalene-2,3-dicarboxaldehyde (NDA, 10 mM; Sigma) with fluorescence readings at 485 (excitation) and 530 nm (emission). SH-gP was estimated as the difference between SH-gT and SH-gNP contents. Results were referred to as the GSH concentration curve, and data were expressed as nmol GSH × 10^6 $cell^{-1}$.
- *Lipid Peroxidation* LPO was estimated by the method of thiobarbituric acid reactive substances (TBARS), according to Hodges et al. (1999). Homogenates were incubated with and without thiobarbituric acid (0.8% w/v of TBA in 0.1% w/v of TCA) and 0.03 butylatedhydroxytoluene (BHT) for 30 min at 90 °C. Reaction tubes were centrifuged at 3000 g for 10 min, and supernatants were measured at 540 nm in a microplate reader. Additionally, a standard curve using tetramethoxypropane (TMP) was run in parallel without BHT addition. LPO was expressed as nanomol of TBARs × 10^6 $cell^{-1}$.

Data Analysis

A one-way ANOVA was performed to detect significant differences among treatments. In cases of statistically significant differences, the mean values were compared with the multiple ranges of Tukey's test. Data were checked to meet the assumptions of normality and homoscedasticity of variances before analysis. Data without normal distribution were analyzed using the Kruskal–Wallis test. Mann–Whitney or *t*-test was used for pairwise comparison. The differences between exposure times were analyzed using a Friedman ANOVA test for dependent samples. The significance level was set at $\alpha = 0.05$.

Results

Cypermethrin Levels in Culture Media

Results showed no effect of experimental conditions on the Cyp levels during 24 h in the control medium (non-inoculated with microalgae) within the rank of 50–500 µg L^{-1}. Under the presence of *P. tricornutum* culture, the Cyp levels were significantly lower than those of the non-inoculated medium ($p < 0.05$; Fig. 1).

Culture Growth

Microalgae growth showed significant differences between treatments in microalgae with or without Cyp or UVR exposure (Fig. 2, $p < 0.05$). Control and UVR cultures showed continuous growth at 24 h with similar values (2.44 ± 0.44 and 2.39 ± 0.26, respectively). The single exposure to Cyp resulted in a significant decrease in growth (= 0.16 ± 0.2, Fig. 2, $p < 0.05$), with high mortalities at T_{24}. However, in the UVR-Cyp treatment, the cultures continued growing (= 1.55 ± 0.08, Fig. 2, $p < 0.05$), compared to the Cyp single treatment at 24 h.

Table 1 UV absorbing compounds (UACs) in *Phaeodactylum tricornutum* cultures from all treatments

	T_h[a]	Co[b]	Cyp[c]	UVR[d]	UVR-Cyp[e]
UACs (DO 10^6 $cell^{-1}$)*1000	T_0	0.92		3.74	
	T_{12}	$1.95^{A} \pm 0.20$	$2.54^{A} \pm 0.28$	$1.05^{A} \pm 0.13$	$2.34^{A} \pm 1.31$
	T_{24}	$0.63^{B\#} \pm 0.24$	$2.06^{A} \pm 0.97$	$0.40^{B\#} \pm 0.25$	$2.09^{A} \pm 0.45$

[a]T_h: exposure time in hours

[b]Co: control, microalgae grown in F/2 media

[c]Cyp: microalgae grown in F/2 media enriched with 0.01 mg/Ltechnical cypermethrin

[d]UVR: Control of microalgae pre-exposed to UVR, grown in F/2 media

[e]UVR-Cyp: microalgae pre-exposed to UVR, grown in F/2 media enriched with 0.01 mg/L technical cypermethrin

[#]Significant differences between time exposure (12, 24 h) within each treatment

*Different upper case letters indicate significant differences in Co/UVR vs Cyp/UVR-Cyp treatments ($p < 0.05$)

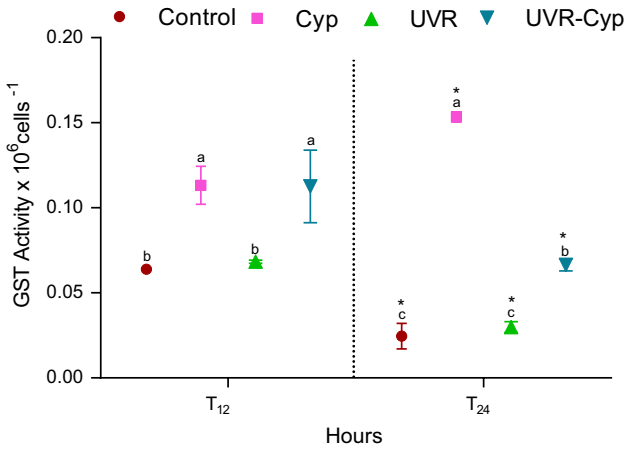


Fig. 3 Glutathione-S-transferase activity (GST activity: nmol × min^{-1} × mg $protein^{-1}$) in *Phaeodactylum tricornutum* cultures at 12 and 24 h (T_h). Control: microalgae are grown in F/2 medium; Cyp: microalgae grown in F/2 medium enriched with 10 µg L^{-1} technical cypermethrin; UVR: microalgae pre-exposed to UVR, grown in F/2 medium; UVR-Cyp: microalgae pre-exposed to UVR, grown in F/2 medium enriched with 10 µg L^{-1} technical cypermethrin. Measurements represent the mean ± SE. At each time, different letters indicate significant differences ($p < 0.05$) in control vs- cypermethrin within UVR pre-exposed or non-pre-exposed treatments. Asterisk indicates significant differences between time exposure (12, 24 h) within each treatment

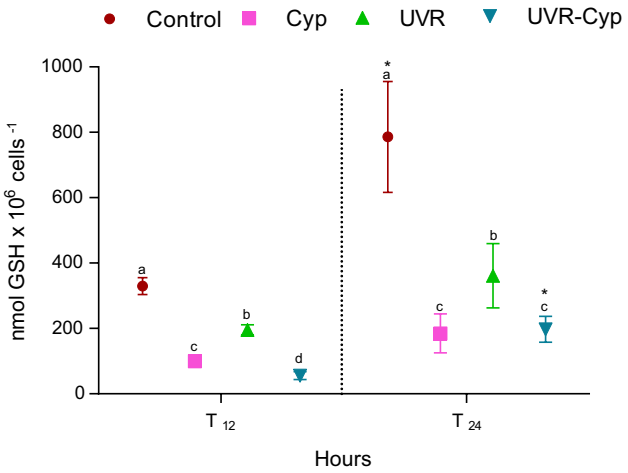


Fig. 4 Total sulfhydryl group (SH-gT) expressed as nmol GSH × 10^6 $cell^{-1}$ in *Phaeodactylum tricornutum* cultures at 12 and 24 h (T_h). Control: microalgae grown in F/2 medium; Cyp: microalgae grown in F/2 medium enriched with 10 µg L^{-1} technical cypermethrin; UVR: microalgae pre-exposed to UVR, grown in F/2 medium; UVR-Cyp: microalgae pre-exposed to UVR, grown in F/2 medium enriched with 10 µg L^{-1} technical cypermethrin. Measurements represent the mean ± SE. At each time, different letters indicate significant differences ($p < 0.05$) among treatments. Asterisk indicates significant differences between time exposure (12, 24 h) within each treatment

UV-Absorbing Compounds (UACs)

In terms of UACs, the UVR cultures showed the highest values (3.74 DO × 10^6 cells*1000) at the beginning of the experiment (T_0, Table 1). Independently of the UVR pre-exposure, a statistically significant decrease in UACs levels was observed in UVR and control treatment at 24 h (Table 1, $p < 0.05$), while Cyp and UVR-Cyp treatments showed no significant changes in UACs levels over time (Table 1, $p > 0.05$).

Oxidative Stress Responses

Cyp exposure led to a higher GST activity than in control, with statistically significant increments between T_{12} and T_{24} (Fig. 3; $p < 0.05$). Regarding differences between exposure times, GST activity in controls decreased significantly at T_{24} in both pre-exposed and non-pre-exposed (Fig. 3; $p < 0.05$). Although GST activity was not affected by UVR (control vs. UVR, Fig. 3; $p > 0.05$), the UVR-Cyp treatment showed significantly higher values than UVR treatment at both sampling times, with significantly decreased activity at 24 h (Fig. 3; $p < 0.05$).

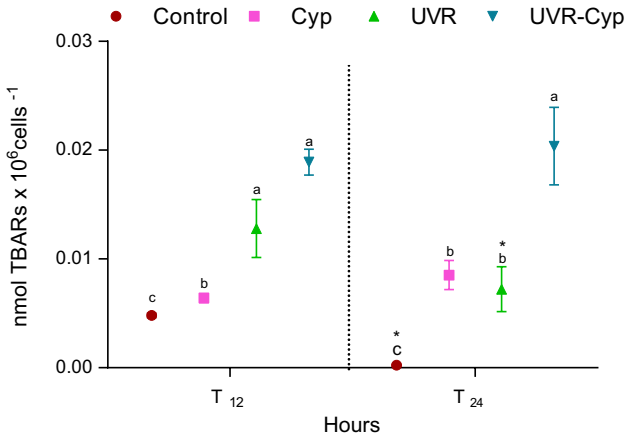


Fig. 5 Lipid peroxidation (LPO, nmol TBARs × 10^6 $cell^{-1}$) in *Phaeodactylum tricornutum* cultures at 12 and 24 h (T_h). Control: microalgae grown in F/2 medium; Cyp: microalgae grown in F/2 medium enriched with 10 µg L^{-1} technical cypermethrin; UVR: microalgae pre-exposed to UVR, grown in F/2 medium; UVR-Cyp: microalgae pre-exposed to UVR, grown in F/2 medium enriched with 10 µg L^{-1} technical cypermethrin. Measurements represent the mean ± SE. At each time, different letters indicate significant differences ($p < 0.05$) among treatments. Asterisk indicates significant differences between time exposure (12, 24 h) within each treatment

Regarding SH groups, all control treatments showed the highest SH-gT content at T_{12} and T_{24} Cyp. UVR-Cyp treatments showed the lowest SH-gT content (Fig. 4; $p < 0.05$). At T_{12}, SH-gT content in UVR treatment was significantly lower than in control, followed by Cyp and UVR-Cyp, which showed the lowest level (Fig. 4; $p < 0.05$). However, the UVR-Cyp treatment showed higher SH-gT content at T 12 than at T_{24}, while Cyp and UVR did not change with time (Fig. 4; $p < 0.05$). When SH groups were discriminated by their protein (SH-gP) or non-protein (SH-gNP) origin, results showed that the observed differences in SH-gT were mainly due to differences in the non-protein groups (Table 2S, Fig. 4S). The combined effect of Cyp and UVR was observed at T_{24} with higher SH-gNP values in the UVR-Cyp than in Cyp and significantly lower SH-gP values (Fig. 4; Table 2S).

Significant LPO level increments at T_{12} were observed in all treatments with regard to control, showing the following pattern UVR = UVR-Cyp > Cyp > control (Fig. 5; $p < 0.05$). After 24 h, LPO levels were significantly lower in control and UVR cultures than at T 12, while no difference with time was observed for Cyp and UVR-Cyp. Moreover, at T_{24}, UVR and Cyp treatments showed LPO levels between control and UVR-Cyp (Fig. 5, $p < 0.05$). LPO increments at T_{24} after Cyp exposure represent about 4000% for the non-acclimatized cultures and about 280% for the control in the UVR-acclimatized cultures (Fig. 5). A graphical representation of the experimental design and the main results is displayed in Fig. 5S.

Discussion

In general, we observed changes at different response levels when *P. tricornutum* was acclimated or not to UVR. The UACs' induction in *P. tricornutum* by UVR exposure was confirmed, and levels at the beginning of the experiment were notable compared to the non-UVR-exposed treatments as was expected for this species (Jeffrey et al. 1999). However, when cultures were no longer exposed to UVR, both control and UVR-acclimatized pre-exposed cultures showed similar UACs' content. This result agrees with Marcoval et al. (2007), who observed the relationship between UVR exposure and UACs' induction in other microalgae species. UACs' content is reduced due to a dilution effect by growth when the *P. tricornutum* cultures were no longer exposed to UVR. However, microalgae exposed to Cyp from UVR- and non-UVR-acclimated cultures exhibited a high accumulation of UACs, suggesting a similar response to both the chemical and environmental stressors (Cyp and UVR) that help to keep constant UACs' levels over time (T_0 to T_{24}). Similar results were found for *Lemna gibba,* where UACs were induced and accumulated after exposure to UVR or increasing copper concentrations under PAR light (Babu et al. 2003).

In terms of Cyp levels, the decreasing concentration of Cyp in the culture medium during the experiment first highlights some considerations related to nominal and observed concentrations when used pyrethroids in microalgae toxic assays. Physical processes like adsorption by cell walls of the microalgae could make the Cyp available to be uptaken and follow biotransformation pathways (Gao et al. 2016). GST mediates the metabolism of xenobiotics by conjugation of glutathione (GSH) to electrophilic xenobiotics. Therefore, the increased GST activity under Cyp treatment in non-UVR-exposed microalgae suggests biotransformation of Cyp by this species. Recent work has shown the increased transcription of GST in the microalgae *Chlorella vulgaris* in response to exposure to Cyp (0.01 µg L^{-1}) at a short time (Gao et al. 2016). UVR acclimation alone did not modify GST activity with time. However, when combined with Cyp, results showed a lower response than in non-acclimatized treatments. Therefore, it suggests a depletion of glutathione intracellular stock due to the UVR pre-exposure. In this sense, cysteine SH-group oxidation in proteins could cause intermolecular cross-linking and enzyme inactivation, among other effects, due to oxidative stress (Ghezzi 2005). All organisms have a regulatory mechanism to keep the redox state of SH-groups (Grant 2001). Data about the influence of UVR or contaminant exposure on SH-g of microalgae are null or scarce. The SH-gT depletion at expenses of the non-protein (SH-gNP) group suggests the use of low molecular weight thiols, like glutathione, to cope

with oxidative stress caused by Cyp (Ahner et al. 2002). The increment in SH-gNP at T_{24} concomitantly with the reduction in SH-gP under UVR-Cyp treatment suggested that some biochemical response might be starting under this condition.

The UVR pre-exposure resulted in higher LPO levels than in non-UVR pre-exposed cultures (Control), and it was related to the known pro-oxidant effect of UVR on microalgae (Liang et al. 2006). Moreover, LPO results were also in agreement with the depleted response of GST activity in UVR-Cyp and showed the additive-like effect of UVR and Cyp. Additionally, Cyp exposure resulted in a significant increment in LPO at T_{12} and T_{24} in non-UVR-acclimated cultures. These results agree with those reported by Wang et al. (2011), where LPO was studied in a co-culture of *Skeletonema costatum*, *Scrippsiella trochoidea,* and *Chattonella marina* after exposure to 50 µg L^{-1} of cypermethrin for 96 h. Sublethal stress responses are early warning signals of possible toxic effects caused by a xenobiotic in the environment. However, in unicellular organisms, growth rate represents a useful endpoint to measure the toxic effects at the population level (Hanson et al. 2019). In this work, Cyp exposure exerted a deleterious effect on the growth rate of *P. tricornutum* cultures, but when microalgae were UVR pre-exposed before being treated with Cyp (UVR-Cyp), the cultures continued growing. These results showed that sub-individual responses measured in a short time are not always in line with population trends.

Besides, considering the work's initial hypothesis, UVR acclimation of *P. tricornutum* could be linked to the tolerance to anthropogenic stressors; it deserves further discussion. The induction of microalgae responses during the UVR pre-exposure as an effective mechanism mitigating the effect of chemical and natural stressors, with similar deleterious effects, was previously reported (Korkaric et al. 2015). Those authors found that UVR-acclimated cultures of *Chlamydomonas reinhardtii* showed co-tolerance to rose bengal but none to the herbicides paraquat and diuron. UVR-induced co-tolerance to Cd was reported at a periphyton community level (Navarro et al. 2008). A typical signaling pathway mediated by oxidative stress responses was proposed for stressors that can generate ROS, independently of the mechanisms (Babu et al. 2003; Korkaric et al. 2015). Therefore, the acclimation to UVR expressed by a high initial content of UACs might be associated with a ROS-mediated signaling pathway that could be linked to other responses triggered during acclimation. The tolerance to Cyp, in acclimated cultures and the maintenance of the UACs content in the non-acclimated cultures upon Cyp exposure, suggests a similar response to both stressors in *P. tricornutum.*

Additionally, some comments should be made regarding the used sub-individual responses. GST, SH-g, and LPO showed a stressor's dependent response, where GST behavior, as a biomarker linked to xenobiotics biotransformation pathways, was only in line with the exposure to Cyp. SH-g response was modified upon the UVR pre-exposure as well as the Cyp exposure. This is of particular interest since studies on SH-g in marine microalgae are scarce, and the GSH stock and its SH-groups are also involved in the tolerance to metals by complexing processes and phytochelatin productions (Pérez Rama et al. 2006; Tang et al. 2000). Therefore, the SH-g could be used to evaluate the influence of different natural and anthropogenic stressors. LPO, like a damage biomarker, was increased by both UVR and Cyp, which was also in line with its widespread use as a valuable oxidative stress endpoint to different stressors (Li et al. 2006; Tsiaka et al. 2013; Lozano et al. 2014; Zhang et al. 2016).

Conclusions

A sublethal concentration of Cyp showed sub-individual toxic effect on *P. tricornutum*, producing evident oxidative burst, as reflected by changes in enzymatic and non-enzymatic responses. GST increment in Cyp exposure was not enough to prevent SH-G depletion and lipid peroxidation. These results confirm the sublethal toxicity of Cyp to non-target species. Also, sub-individual effects on UVR-acclimated microalgae suggest additive effects of chemical and environmental stressors, highlighting the potential hazard upon simultaneous occurrence. However, at a population level, the observed continuous growth of UVR-acclimated cultures under Cyp exposure suggests other compensatory mechanisms that counteract the toxic effects of pyrethroid insecticides. Our results revealed different interaction scenarios depending on the biological-level responses assessed regarding the combined effect of natural and chemical stressors. These findings also revealed the importance of including both sub-individual and population responses to avoid misinterpretations in assessing the effect of multiple stressors on marine microalgae. These findings revealed the complexity in assessing the effects of pesticides in natural/realistic scenarios where microalgae inhabit. Finally, considering that *P. tricornutum* only represents one of the several phytoplankton species with the ability to induce UACs upon UVR exposure, these findings could be extrapolated to similar microalgae species. Future works will focus on revealing whether the observed interactions might also be present in other species or upon other chemical stressors.

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Author's contributions JC was involved in investigation, formal analysis, and writing—original draft. Ma. AM performed resources, investigation, and writing—original draft. MD-J contributed to resources, writing—review and editing, visualization. MG done conceptualization, supervision, writing—review and editing, visualization, funding acquisition.

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Data Availability Supplementary information is available.

Declarations

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

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