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Toxicity of a Commercial Sunscreen Combined with Temperature Stress: Impacts on Growth and Cellular Oxidative Balance in Raphidocelis subcapitata

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Abstract

Sunscreens contain active ingredients known as ultraviolet (UV) filters, which can be either organic or inorganic. These UV filters play a crucial role in protecting skin tissue against the harmful effects of UV rays. Nonetheless, their presence can significantly impact biotic communities, particularly microalgae. The aim of this study was to evaluate the effect of different environmentally relevant concentrations (0, 5, 10, 25, 50, 100, and 200 mg L-1) of a commercial sunscreen on the growth of Raphidocelis subcapitata, given two temperature scenarios. Cultures were maintained under controlled conditions in a climatic chamber, at 24°C and 29°C, with continuous agitation throughout the 96h exposure period. In this study, flow cytometry analysis was used to measure growth and Reactive Oxygen Species (ROS) production, while chlorophyll levels were assessed via autofluorescence. The growth of R. subcapitata was significantly inhibited in all sunscreen treatments compared to the control, irrespective of the temperature tested. ROS production was detected for all concentrations tested. Notably, treatment with 10 mg L-1 resulted in a significant increase of ROS, with levels rising by 26% at 24°C and 41% at 29°C. Additionally, at 24°C, chlorophyll a fluorescence significantly decreased ($p < 0.05$) in R. subcapitata when exposed to 200 mg L-1 of sunscreen compared to the control, whereas no significant change was observed at 29°C. These findings reveal the detrimental impact of commercial sunscreens on Raphidocelis subcapitata growth and ROS production, independent of temperature variations. This highlights the significant ecological concerns associated with sunscreen ingredients. Further research is necessary to understand long-term impacts and develop sustainable solutions.

Highlights

- Increased sensitivity of *Raphidocelis subcapitata* exposed to a commercial sunscreen was temperature dependent (IC_{50} 23.85 mg L^{-1} at 24°C; 12.37 mg L^{-1} at 29°C).
- At the studied concentrations, the tested sunscreen had a negative effect on growth and Chlorophyll fluorescence of *R. subcapitata.*
- Sunscreen exposure affected morphology and structure of *R. subcapitata.*
- Sunscreen ingredients had a greater effect on ROS production for *R. subcapitata* at 29°C, compared to 24°C.

1. Introduction

Aquatic ecosystems are continuously affected by a wide variety of anthropogenic activities, including the use of sunscreens. Sunscreen products, classified within the skincare products group, can directly or indirectly introduce a multitude of chemical compounds into the environment (Ruiz-Gutiérrez et al. 2022). Formulations contain organic ultraviolet (UV) filters called active chemical components and/or inorganic UV chemical filters, which absorb and stabilize solar UV radiation in the range of UV-A, UV-B, or both (Tovar-Sánchez et al. 2013). Inorganic UV filters mainly include zinc oxide (ZnO) and titanium dioxide (TiO_2), while organic ones contain 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC), benzophenone-3 (BP-3), 4-methyl-benzylidene camphor (4-MBC), octocrylene (OC) and methyl anthranilate (Ruszkiewicz et al. 2017). In recent years, their presence has been frequently detected in wastewater treatment plants, surface waters, groundwaters, lakes, and sediment deposits (Zhang et al. 2015; Mitchelmore et al. 2019) due to their lipophilicity and photostability in water environments (Zucchi et al., 2011).

Surface water is perhaps the least contaminated compartment by UV filters, with reported concentrations ranging from 0.5 to 6812 ng L^{-1} (Balmer et al. 2005; Fent et al. 2010; Tovar-Sánchez et al. 2013; Ruiz-Gutiérrez et al. 2022). Median concentrations of UV filters were 250 ng L^{-1} found in marine waters near public beaches with high sunscreen usage (Tsui et al. 2014). Contamination of aquatic biota provides evidence of bioaccumulation across the food chain. For example, in Switzerland and Germany UV filter compounds such as 4-MBC (4-methylbenzylidene camphor), BP-3 (benzophenone-3), EHMC (ethylhexyl methoxycinnamate), and OC (octocrylene) have been detected in fish from rivers and lakes (Balmer et al. 2005; Fent et al. 2010).

In algae, UV filter compounds have been demonstrated to adversely affect both photosynthetic and mitochondrial electron transport, while some of these chemicals can inhibit algal growth at concentrations as low as 10 ng L^{-1} (Mao et al. 2017; Zhong et al. 2019; Míguez et al. 2021). Exposure to these UV filters has also been associated with reductions in chlorophyll content, alterations in cellular morphology, and induction of reactive oxygen species (ROS) production (Ajitha et al. 2021). An excess in ROS can disturb redox homeostasis and damage cell components in microalgae, leading to an induction of cell death and antioxidants or reactive nitrogen species, among others (Mao et al. 2017; Fal et al. 2022).

As primary producers, microalgae are important organisms in toxicity tests due to their environmental relevance, extensive distribution and fast growth rate (González-Pleiter et al. 2019). In ecotoxicology, current standard toxicity tests with microalgae are usually carried out with monoalgal cultures (Seoane et al. 2021). Growth inhibition is a relevant endpoint to test microalgal toxicity, and it is frequently used to observe the toxicity over multiple algal generations (Prado et al. 2012).

In all ecosystems contamination by chemicals rarely occurs in isolation, there is a growing concern regarding the effects of multiple simultaneous environmental stressors on microalgae (Hernando et al. 2018). Physical stressors such as temperature may amplify the adverse impacts of compounds commonly found in the formulation of sunscreens by altering their bioavailability or toxicity to microalgal communities (Wang et al. 2021; Amaro et al. 2023). Temperature fluctuations can alter algal growth and all the biochemical reactions that occur in algal cells, but also, increasing temperatures may boost overall productivity, resulting in an increment of undesirable species and decreases in biodiversity (Kholssi et al. 2024b). Thus, it is important to understand how formulations of commercial sunscreens and temperature could jointly affect aquatic microalgae. Such interactions are particularly relevant in the context of climate change, and it is important to understand the response of microalgae in such circumstances.

To address this knowledge gap for freshwater and tropical scenarios, we hypothesize that the interaction of temperature and sunscreens could cause deleterious effects in *Raphidocelis subcapitata* during short-term exposures. The objective of this study was to assess the toxicity of six concentrations of a

commercial sunscreen under two temperature levels 24 and 29° C on the freshwater microalgae *R. subcapitata*. Analyses were conducted using flow cytometry to evaluate three parameters: growth rate, oxidative stress and viability of *R. subcapitata*.

2. Methodology

2.1. Microalgae culture

R. subcapitata formerly known as *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata* (Machado and Soares 2024) inoculum was obtained from an axenic stock culture in the exponential growth phase, maintained under controlled conditions at the Laboratory for Ecotoxicological Studies (ECOTOX) of the Central American Institute for Studies on Toxic Substances (IRET), Universidad Nacional, Costa Rica. The stock culture was exposed to illumination of 4,500-5,000 lux with a 16:8-hour light: dark photoperiod, and maintained at a temperature of 24 ± 2°C. To prevent sedimentation, the culture was manually agitated three times daily, while continuous aeration was provided to ensure uniform nutrient distribution and homogeneous light exposure.

The algae were maintained in a 5X culture medium prepared using ultrapure Milli-Q water, supplemented with essential macronutrients and micronutrients, including nitrogen and phosphorus, following the concentrations specified by the EPS 1/RM/25 protocol (Environment Canada, 1992). The medium was sterilized before use to prevent microbial contamination. One week before the test, a subculture was prepared by inoculating 1–2 mL of the stock culture (in the exponential growth phase) into 100 mL of fresh, sterile 18X medium and maintained following the guidelines of Canadian Environmental Assessment (CEAA 1992 |)to ensure the inoculum was in optimal physiological condition for the bioassay.

2.2. Commercial sunscreen test solutions and assay

An over-the-counter commercial sunscreen was selected for the assay based on its composition (with octocrylene as the main active ingredient). Six concentrations of a formulated commercial sunscreen were tested (5, 10, 50, 100 and 200 mg L^{-1}) to assess the toxic effects of Octocrylene on *R. subcapitata* (Table 1**).** Test solutions were prepared by weighing specified amounts of sunscreen on an analytical balance (Sartorius CPA224S) using aluminum foil capsules. For each solution, the weighed amount of sunscreen was dissolved in 150 mL of 5X algal medium, which is routinely used at the ECOTOX Lab for the algal maintenance, according toguidelines for Exposure Assessment (EPA 1992) and (CEAA1992). The 5X medium also was used as negative control. The final concentrations of sunscreen in each test solution were as shown in Table 1. Each concentration was tested in triplicate in 100 mL Erlenmeyer flasks containing 50 mL of the solution. Algae were inoculated in each flask to achieve an initial cell density of ≈ 10^4 cell mL^{-1}. This concentration was verified by measuring cell density using a flow cytometer (conditions below). All replicates were incubated for 96 hours in a LAB Companion ISS 4075 incubator, under continuous light (4000 lux) and constant shaking (100 rpm). One set of concentrations was incubated at 24°C, and another at 29°C (Table 1). The organisms involved in the toxicity tests were monitored by using flow cytometry (FCM) at 0, 48, 72, and 96h of exposure.

Table 1
Nominal sunscreen concentrations in each test solution and treatment

Treatment code	Concentration (mg L^{-1})	Weight (mg) at 24°	Weight (mg) at 29°C
SSC1	5	5.73	4.80
SSC2	10	11.40	11.60
SSC3	25	27.00	26.67
SSC4	50	50.47	53.67
SSC5	100	100.87	99.87
SSC6	200	210.20	196.00
Control (Ct)	0	0	0

2.3. Flow cytometry and cell analysis

At each time, 2 mL samples were taken from each replicate, filtered using test tubes with a 35 µm cell strainer (Falcon™), and analyzed using a BD Accuri C6 Plus flow cytometer (FCM) (Becton Dickinson) equipped with a 488 nm blue excitation laser and a 640 nm red laser, and calibrated using BD™ CS&T RUO Beads. C6 Plus Analysis Software (BD) was used during acquisition and analysis. The following parameters of each event were recorded: forward scatter and side scatter, red fluorescence related to chlorophyll a (FL3, > 670 nm emission after blue light excitation), and green fluorescence (FL1, 533/30 nm emission after blue light excitation). FL3 vs FSC cytograms were used for cell gating and enumeration, while histograms of gated cells were analyzed in FL1 for oxidative stress and cell viability tests. The samples were mixed via manual shaking for 10 s before running FCM. FCM parameter settings were: flow rate 66 µl min-1, sample volume of 25 µL, and washing volume 100 µL. The specific growth rate (SGR) was calculated as the slope of the regression line from a plot of log10 (cell density) versus time over 96h (Fogg and Thake (1987).

$$\mu = \ln\left(\frac{N2}{N1}\right) \ (t2 - t1)$$

where, µ represents the SGR (day^{-1}), while N1 and N2 are the cell concentrations at times t_1 and t_2, respectively.

2.4. Morphological changes

At 96h, all samples were photographed at (40x magnification) using an inverted microscope (Nikon, Eclipse Ts2, Japan) coupled to a digital camera (Nikon, DS-Ri2, Japan). Microscopic examination aimed to characterize curved-shaped cells typical of *R. subcapitata*. Any deviations from the standard morphology, such as cell elongation, aggregation, or deformation(Machado and Soares 2024) were noted as indicators of physiological stress by sunscreen and temperature.

2.5. Chlorophyll fluorescence

Chlorophyll *a* fluorescence was monitored using fluorometric plate-reader (Thermo Scientific™ Varioskan™ LUX Multi-Mode Microplate Reader) in 96 well white flat-bottom plates at room temperature, in 300 µL of volume each well, by quadruplicate and with linear and orbital shaking during 3s. Fluorescence was measured at a fixed gain value of 60 using excitation-emission filters of 360–670 nm, respectively.

2.6. Oxidative stress measurement: determination of intracellular levels of reactive oxygen species (ROS)

Intracellular reactive oxygen species (ROS) were measured at the end of the experiment to detect oxidative stress. The method of (Stachowski-Haberkorn et al. 2013) was employed to quantify ROS production using the 2'-7'-dichlorofluorescein di-acetate (DCFH-DA) (Invitrogen Molecular Probe, USA). H_2DCFDA (non-polar compound) diffuses into algal cells where it is de-acetylated by esterases and H_2O_2 into fluorescent 2',7'-dichlorofluorescein (polar), which has a maximum emission between 517 and 527 nm (Perreault et al. 2012), using FCM.

Test Solutions:

Preparation of Stock Solution (SS) 10 mM: A total of 9.74 g of DCFH-DA was weighed into a sterile 15 mL beaker, and 2 mL of 99.5% dimethyl sulfoxide (DMSO) was added. The mixture was stirred until fully dissolved.

Preparation of Phosphate Buffered Saline (PBS): In a 50 mL beaker, 2.742 mg of KCl and 1,35 mg of NaCl were dissolved in 30 mL of ultrapure water (Milli-Q). The solution was then brought to a final volume of 50 mL in a 50 mL volumetric flask. The pH was adjusted to 7.65 using NaOH and HCl solutions.

Preparation of 20% Working Solution (WS): To the 2 mL stock solution (SS), 8 mL of PBS solution was added and stirred for 10 min. This resulting working solution was stored in a 10 mL conical plastic tube, completely covered in aluminum foil to prevent light exposure.

Final Working Solution (0.8%): This solution was used to assess oxidative stress in the algal cells. At 96 hours of exposure, 480 µL of sample and 20 µL of the working solution (WS) were added to a flow cytometry tube fitted with a 35 µm. The mixture was incubated for 30 min in the dark at room temperature. Oxidative stress was assessed by measuring the release of free radicals using a flow cytometer.

2.7. Cell viability test with SYTOX-Green

For each sample at 76 hrs, cells were stained with SYTOX-Green (Molecular probes, Eugene, OR, USA), which permeates membranes of dead cells only (Machado and Soares 2024). SYTOX Green is provided in DMSO and prepared in at a concentration of 5 mM for stock and kept at -20°C. A working solution in ultrapure water at 5 µM was prepared from stock, to stain cells at final concentration 0.5 µM (-360µL of sample and 40 µL of working solution). A heat-treated positive control was prepared by incubating a control sample at 60°C for 60 minutes prior to staining. Thus, by counting the cells stained by SYTOX-Green it was possible to estimate the percentage of dead cells in each sample (Veldhuis et al., 2001). Samples were incubated in the dark at 25°C for 30 min before FCM analyses. Thermal death by microwave heating was included as positive control of the test. The evolution of the staining SYTOX-Green of the algal cells was monitored by measuring the emission of green fluorescence (FL1) by FCM. *R. subcapitata* was gated on FL3 vs FSC plots, and membrane-compromised cells were identified by increased green fluorescence intensity in the FL1 channel. Gating thresholds were defined based on negative (unstained and SYTOX-stained) and positive (heat-damaged) controls. The percentage of SYTOX Green-positive cells was reported as an indicator of membrane damage.

2.8. Data Analysis

The area under the curve (AUC) described by algal growth curves (cell µL vs time in hours) was used for estimating growth inhibition, and comparison among concentrations and temperature treatments, because it provided integrative and objective data regarding the whole behavior of the culture in the time period analyzed. AUCs were estimated by the trapezoid function in RStudio. In all parameters used in this experiments, statistical differences between temperatures and concentrations were analyzed by one way ANOVA after checking Shapiro–Wilk's and Levene's tests ($p > 0.05$) to confirm normal distribution and homoscedasticity, respectively. The data with non-normal distribution or unequal variance were analyzed using the Kruskal-Wallis test and non-parametric Mann-Whitney U test. Statistical significances were considered for $p < 0.05$ level. A correlation matrix-based principal component analysis (PCA) was used to determine the correlation between analyzed parameters (R). Statistical analyses and graphs were performed using IBM SPSS version 22.0; PriProbit was used as tool to determine growth inhibition as estimated median effective concentration (IC_{50}).

3. Results

3.1. Growth of *R. subcapitata*

The specific growth rate (SGR; d^{-1}) of *R. subcapitata* was significantly affected by both temperature and sunscreen concentrations. At both 24°C and 29°C, a clear concentration-dependent decrease in SGR was observed with increasing concentration of sunscreen from 5 to 200 mg L^{-1}. At 24°C, Kruskal Wallis-test ($H(6) = 19.64$, $p = 0.03$) revealed that the control significantly maintained higher growth rates (1.34 ± 0.01 d^{-1}) than all exposed treatments (Fig. 1).

SGR were impacted more by the highest concentration tested in 200 mg L^{-1} (0.52 ± 0.10 d^{-1}) compared to control and all other exposed treatments. The results exhibited that even the lowest concentration of 5 mg L^{-1} decreased SGR significantly compared to control. Similarly, at 29°C, one-way analysis of variance (ANOVA) revealed a statistically significant effect of sunscreen concentration on SGR of *R. subcapitata* ($F(6,14) = 205.553$, $p < 0.001$). Control exhibited the highest SGR (1.54 ± 0.004 d^{-1}), significantly higher ($p < 0.05$) than all sunscreen treatments. SGR decreased progressively with increasing concentration from 5 to 200 mg L^{-1} (Fig. 1).

In Fig. 2, the toxicity of the sunscreen on *R. subcapitata* at 24 and 29°C showed a significant result. Growth inhibition at 24°C remained low at the lowest concentrations tested (5 and 10 mg L^{-1}). However, toxicity increased at 50 mg L^{-1}, reaching 55% and continued to increase until 69% of inhibition at 100 mg L^{-1}. A maximum growth inhibition at 200 mg L^{-1} was observed, reaching 87%. A similar concentration-dependent pattern was observed at 29°C, with overall higher levels of growth inhibition compared to 24°C. Inhibition increased from 5 to 200 mg L^{-1}, reaching the same value of the lowest temperature tested in this study. The difference in inhibition between 24°C and 29°C appeared more marked at intermediate concentrations (50 and 100 mg L^{-1}), suggesting that elevated temperature may enhance the toxic effects of sunscreen at these levels.

Besides that, our results showed that the estimated median growth inhibition concentration (IC_{50}) due to sunscreen exposure in *R. subcapitata* was 23.85 mg L^{-1}at 24°C (95% CI: 17.79–31.16 mg L^{-1}), and 12.37 mg L^{-1}at 29°C (95% CI: 7.99–17.18 mg L^{-1}), showing a reduction in the IC_{50} value with increasing temperature.

3.2. Morphological changes

R. subcapitata morphology was examined under each treatment condition **(Figure S.P 1**). After 96 h of exposure to temperature of 24°C without sunscreens, cells in control maintained their normal shape, and dispersed independently. Similarly, cells exposed to sunscreen concentrations ranging from 5 to 25 mg L^{-1}at 24°C appeared in their normal morphology. However, cells showed signs of elongation from 50 to 200 mg L^{-1}, sticking together and forming aggregates especially in the highest concentration of sunscreen (indication in blue color Fig. 3.

The morphology of untreated cells was intact and crescent-shaped at 29°C, while morphological changes in sunscreen-treated cells were clear (Fig. 3). Visible individual cells started to change their shape in concentrations SSC1 to SSC6. Exposure to sunscreen resulted in deformation, elongation, and decreases in cell size (indication in red color Fig. 3). Some cells exhibited visible holes at the highest concentration tested and some cell residues were observed (not shown in the figure), indicating rupture of the cell and evident signs of structural damage.

3.3. Chlorophyll fluorescence in *R. subcapitata*

The autofluorescence patterns of chl-fluorescence in *R. subcapitata* culture at 24 and 29°C under exposure of sunscreen from 0 to 200 mg L^{-1} is presented in Fig. 4. The effect of temperature resulted in a significant decrease in chl-fluorescence at 24°C from 25 to 200 mg L^{-1} with respect to control, while the lowest concentrations tested (5 and 10 mg·L^{-1}) exhibited a marked increase in this parameter. At 29°C, one-way ANOVA showed statistically significant effect of sunscreen concentrations on chl-fluorescence $F(6, 14) = 108.51$, $p < 0.001$. The presence of sunscreen triggered chl-fluorescence from 5 to 50 mg L^{-1} then decreased at 100 and 200 mg leading to a significant decline in chl-fluorescence, particularly at the highest concentration tested.

3.4. Oxidative stress measurement: determination of ROS

Results of diclofluorescein diacetate test performed on microalgae under exposure to six concentrations (5–200 mg L^{-1}) and two temperatures (24 and 29°C) for 96h are reported in Fig. 5. Oxidative stress biomarkers revealed an increase in ROS levels at both temperatures tested, showing a significant ($p < 0.001$) increase in ROS level at 29°C compared to 24°C. Exposure to a temperature of 29°C alone induced the production of 90 ± 0.83% ROS in control compared to all treatments. In the presence of sunscreen, concentrations of 50 and 200 mg L^{-1} showed the next highest ROS levels, while the lowest ROS production was observed with 5 and 10 mg L^{-1}. Whereas, at 24°C, ROS levels were generally lower, reaching 26 ± 3.30% in treatment SSC2 and significantly declining from SSC3 to SSC6. The control at 24°C showed the lowest ROS level 15%, statistically different from most sunscreen treatments.

3.5. Cell viability

Cell viability of *R. subcapitata* cultures, assessed by FCM, was significantly affected by exposure to sunscreen at 29°C ($p < 0.05$; Fig. 6). The positive control (Ct^{+}), which induced maximum membrane damage, showed the highest fluorescence intensity, indicating extensive loss of membrane integrity. In contrast, the negative control (Ct) exhibited minimal fluorescence, consistent with intact and viable cells. No significant differences in fluorescence were observed between the negative control and the low-dose sunscreen treatments (5 and 10 mg L^{-1}; $p > 0.05$), suggesting minimal cytotoxicity at these concentrations. Intermediate sunscreen concentrations (50–100 mg L^{-1}) caused a moderate but significant increase in cell death, while the highest concentration tested (200 mg L^{-1}) led to a pronounced loss of membrane integrity, reflected by elevated SytoxGreen fluorescence. The assay results for temperature 24°C were excluded from analysis due to irregular responses in the negative control, which made the data unreliable.

3.6. Multivariate analysis of physiological and biochemical responses

Figure 7 provides complementary insights into the relationships among physiological and biochemical parameters under two temperatures 24 and 29°C and exposure of sunscreen from 0 to 200 mg L^{-1}. The correlation heatmap (Fig. 7a) shows that elevated ROS levels, particularly under stress, are strongly and negatively correlated with chl-fluorescence, AUC, and SPR, indicating oxidative damage and reduced physiological function. In contrast, chl-fluorescence is positively associated with AUC and SGR, indicating these parameters reflect stable photosynthetic activity. The PCA biplot (Fig. 7b) emphasizes these

patterns, with ROS at 29°C and porcentage of inhibition contributing most to variance along PC1, separating stressed treatments (e.g., SS4–SS6) from controls and less affected samples. Meanwhile, chl-fluorescence and ROS at 24°C load in opposing directions, highlighting their inverse relationship with stress markers. Together, these analyses demonstrate that ROS accumulation is closely linked to physiological decline, while photosynthetic measure serve as indicators of resilience.

4. Discussion

4.1. Growth of *R. subcapitata*

The results of this study demonstrate that both temperature and sunscreen exposure significantly affect the growth rate of *R. subcapitata*, with interaction effects observed between these stressors. Specifically, growth inhibition increased progressively with increasing sunscreen concentrations, and this effect was further augmented at 29°C. This indicates a synergistic interaction between chemical (i.e., sunscreen ingredients) and physical stress, indicating the vulnerability of microalgae to combined environmental stressors. At both selected temperatures, SGR declined as concentration of sunscreen was increased, with the highest inhibition observed at the maximum sunscreen concentration (200 mg L^{-1}). Even at the lowest tested concentration (5 mg L^{-1}), a statistically significant reduction in growth was observed, demonstrating the high sensitivity of *R. subcapitata* to the tested sunscreen formulation. These findings agree with Li et al., 2024, who also reported a reduction in growth rates of *S. capricornutum* with increasing concentrations of benzophenone-3 (OBZ). At 29°C, SGR was generally higher in control but decreased more quickly with sunscreen exposure compared to 24°C, particularly at concentrations between 50–100 mg L^{-1}. Higher toxicity at elevated temperature may be due to increased metabolic rates, which could raise cellular uptake of toxicants or exacerbate stress responses, as also proposed in Clergeaud et al., 2025 for a group of microalgae exposed to different UV filters.

The observed pattern of growth inhibition in Fig. 2 supports the SGR findings. Growth inhibition at 24°C increased moderately from 55% at 50 mg $L^{-1}$to a maximum of 87% at 200 mg L^{-1}. At 29°C, similar maximum inhibition was observed, but with greater toxicity at mid-range concentrations, reinforcing the idea that temperature exacerbates the toxicity of sunscreen contaminants. This aligns with Schiavo et al., 2018), who reported that sunscreen nanoparticle extracts inhibited algal growth within 24 h, with partial recovery over time, suggesting initial acute effects may be followed by cellular adaptation, though such recovery may be suppressed at elevated temperatures. Walton, 2018studied the fluorescence of chlorophyll as a parameter of growth inhibition of 4 common UV filters found in cosmetic care products on *Scenedesmus acutus*. The results of this work demonstrated that all UV filters inhibited growth with increasing concentration, except for Avobenzone and Octisalate, which did not decrease reproduction at any treatment level up to water solubility. Atrazine, Oxybenzone, and Homosalate were 117, 1875, and 100 µg L^{-1}. Moreover, the present study supports findings by Yang et al., 2024, who found that certain benzophenone-based UV filters (e.g., BP3) exhibited high toxicity through inhibition of photosynthesis, reduction of cell viability, and damage to membrane integrity. Similar results were observed in the same species used in our experiment when assessing the impact of CeO_2. The study demonstrated that particulate toxicants could elicit toxic responses either through direct interaction with the cell membrane or following cellular uptake. This exposure resulted in a 50 % reduction in algal growth rate after 72 hours (C_{50}), with values of 10.3 ± 1.7 mg $L^{-1}$for nanoparticles and 66 ± 22 mg $L^{-1}$for bulk materials, respectively ((Rogers et al. 2010a).

4.2. Morphological changes

R. subcapitata maintained their normal shape and dispersed independently under control conditions. However, cells showed signs of elongation under sunscreen concentration from 50 to 200 mg L^{-1}, sticking together and forming aggregates especially at 200 mg L^{-1} at temperature of 24°C. It appears that at least under short term exposure visible individual cells started to aggregate together probably due to the amount of extracellular organic matter secreted by *R. subcapitata* in response to unfavorable growth conditions. Under toxicological effects of microplastics (Mps) and Sulfadiazine on *Chlamydomonas reinhardtii*, cells exhibited aggregation with Mps (Li et al. 2022).

Exposure to sunscreen resulted in deformation, elongation and decreases in cell size at 29°C. An alteration from the typical lunate shape to French croissant type (Fig. 3B) was reported when the algae has been exposed to inorganic or organic pollutant (Machado and Soares 2024). Probably, the observed effect is due to the penetration of the chemicals inside the cells that provoke mechanical damage to intracellular organelles such as chloroplasts, mitochondria, and nucleus (Shoman et al. 2024) in particular chloroplasts. The morphology of untreated *R. subcapitata* cells persisted intact with curved shape compared to cells exposed to nanoparticles (ZnO NPs) which exhibited clear morphological alterations, with some cells showing complete disintegration and damage even lost in chlorophyll, resulting in cellular debris(Samei et al. 2019).

4.3. Chlorophyll fluorescence in *R. subcapitata*

The effects of temperature induced a significant decrease in chl-fluorescence intensity at 24°C from 25 to 200 mg L^{-1} with respect to control, while the lowest concentrations (5 and 10 mg L^{-1}) exhibited a slight increase (Fig. 4), possibly indicating a hormetic or stimulatory effect at low exposure levels (Kholssi et al. 2024a), potentially induced by one or more components present in the sunscreen formulation. Similarly, the presence of sunscreen triggered Chl-fluorescence till concentration of 50 mg L^{-1} at 29°C, then decreased at 100 and 200 mg L^{-1}. Chl- fluorescence is a key indicator of the physiological status of microalgae, because it provides information on energy in photosynthesis such as absorption, distribution and utilization (Hawkins and Griffiths, 1982). These results suggest that while low concentrations of a sunscreen may quickly enhance photosynthetic activity, higher concentrations of sunscreens under elevated temperature can reduce it. This reduction in Chl-fluorescence might be a response to the exposure to chemicals present in this sunscreen, since inorganic and organic UV filters contained in these formulations have been proven to disrupt the synthesis of photosynthetic pigments in marine primary producers, such as macro(García-Márquez et al. 2023) and microalgae (Schiavo et al. 2018; Li et al. 2024). According to Yang et al., 2024, an altered pathway of photosynthesis after exposure to three different benzophenone especially at 0.1 TU of BP3 in *Chaetoceros neogracilis*. Under stress, combined effects of cobalt and nickel on the microalga *R. subcapitata* showed significant increases by some concentrations and decreases in this parameter in other concentrations (dos Reis et al. 2024).

4.4. Oxidative stress measurement: determination of ROS

The sunscreen formulations, in combination with temperature, significantly induced oxidative stress in *R. subcapitata*, as shown by the increased percentage of cells (ROS) (Fig. 5). The highest ROS production at 24°C was observed at 10 mg L^{-1}, whereas the highest value of ROS production at 29°C was obtained in control followed by 200 mg L^{-1}, considering that even low to moderate concentrations of sunscreen can provoke a synergistic effect, inducing an oxidative stress in algal cells. Temperature is a critical environmental factor that influences ROS levels, inducing oxidative stress at high temperature (Koletti et al. 2025). Our findings align with previous studies of(Li et al. 2024) who found that oxybenzone which is considered as an organic compound used in sunscreens and light stress, conduced to an over-accumulation of ROS resulted in the peroxidation of cytomembranes. Similarly, ROS were increased after exposure to 10, 20 and 50 mg L^{-1} of N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6 PPD), demonstrating an imbalance in the antioxidant system (Yan et al. 2024).Schiavo et al., 2018also observed that sunscreen induced ROS production in 75 % of cells linked to the ability to penetrate through cell membranes and hrough activation of irradiation mechanisms of TiO_2 nanoparticles or organic ingredients. In this study, the generation of ROS is a major reason behind the toxic effects of the sunscreen combined with the effect of temperature. According to literature data, as a result of oxidative stress, some chemical components such AuNRs often damage mitochondrial functions and induce cell death(Zhang et al. 2016) .

4.5. Cell viability

The percentage of cells with decreased viability under sunscreen exposure was significantly different between treatments at temperature 29°C. The percentage of cells with increased permeability was generally low (~ 10–15%) across most sunscreen concentrations at this temperature (compared to positive control). However, percentage of cells with increased permeability under 200 mg L^{-1} remained high compared to control, confirming its persistent cytotoxic effect. The results at temperature of 24°C are not considered representative, as the positive control for this condition failed due to the same value found it as our samples, the validity of the data under these conditions is therefore invalidated. In a study by (Nogueira et al. 2020), the decreases in cell viability were significant for gold nanorods with higher aspect ratio 3.50 at a concentration of 10 µg mL^{-1}. This effect was probably caused by damage in the metabolic mechanism and cellular structure of the cells, in response to the oxidative stress. The effects of sunscreen ingredients on microalgae may involve changes in their physiological and biochemical states, which occur due to distinct types of interactions in algal cells such as peroxidation and damage in membrane lipids which could initiate a surface disruption (Rogers et al. 2010a). *R. subcapitata* exposed to nanoparticulate CeO_2 were permeable to the DNA-binding dye SYTOX® Green in a concentration-dependent manner indicating damage to the cell membrane (Rogers et al. 2010b).

The results generated in the combined analysis of cell viability through SYTOX-Green fluorescence and oxidative stress revealed clear temperature-dependent responses of algal cells to sunscreen exposure. Our cytometric analysis at 29°C showed that SYTOX-Green signals were lower among all treatments exposed to sunscreen, except for 200 mg L^{-1}, implying improved membrane integrity or a stress-adaptive cellular response at high level of temperature. This was unanticipated, as higher temperatures often amplify toxicity.

5. Conclusions

Our results demonstrated that sunscreen and temperature (24 and 29°C) altered the metabolism of *R. subcapitata* and showed that a synergistic interaction can occur depending on the concentration. Both stressors tested affected SGR, Chl-fluorescence, intracellular ROS production and cell viability, generating key structural and physiological changes related to chemical (sunscreen ingredient) and physical (temperature) toxicity. This highlights the importance of investigating the toxic effects of different sunscreens on freshwater microalgae, as the combined effects of multiple ingredients may differ potentially enhancing or reducing toxicity compared to single components. Ecotoxicological investigations should consider the association between sunscreen concentrations and other parameters of culture conditions to provide more realistic data of the direct effects in microalgae communities and indirectly in primary consumers that depend on the microalgae in natural water.

Declarations

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Author Contributions

Kholssi R., Conceptualization, Methodology, Investigation, Assay execution, Formal analysis, Software, Visualization, Writing - original draft. **Arias-Andrés, M.**, Conceptualization, Investigation, Assay execution, Formal analysis, Writing - review & editing. **Echeverría-Sáenz, S.**, Conceptualization, Assay execution, Formal analysis, Writing - review & editing. **Ugalde-Salazar, R.**, Funding acquisition, Investigation, Assay execution, Writing - review & editing.

Ethical Approval

This is not applicable

Consent to Participate

This is not applicable

Consent to Publish

This is not applicable

Competing Interests

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

Data Availability Statement

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Figures

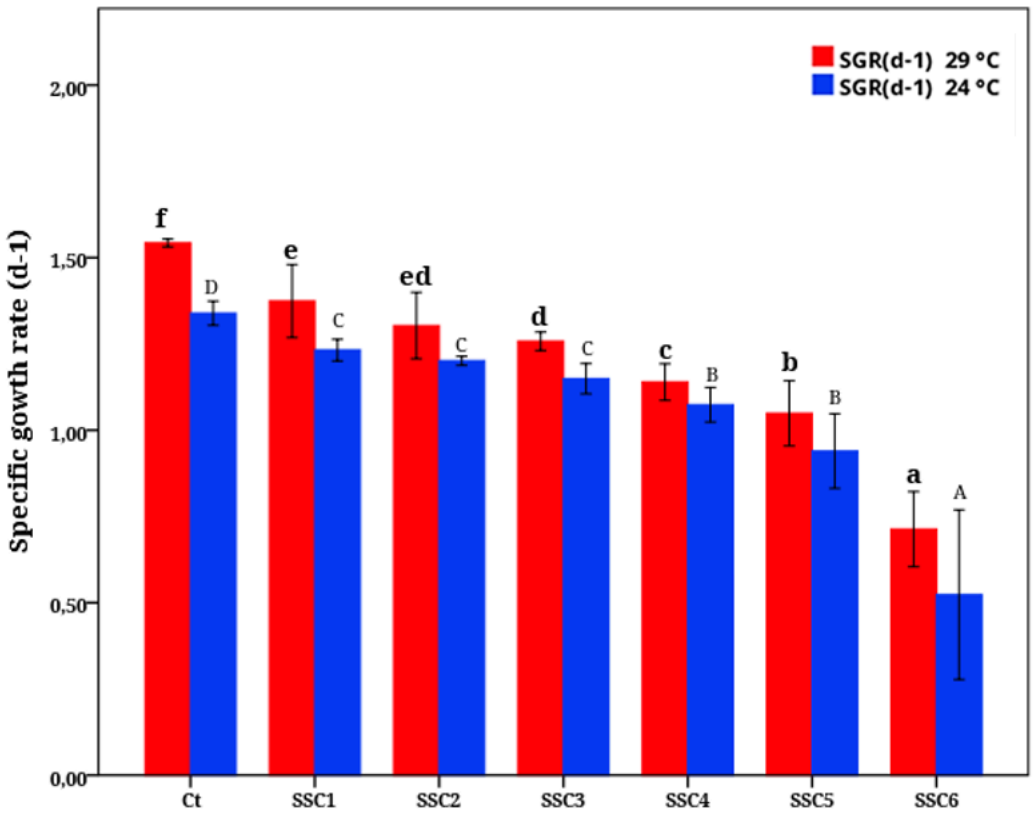


Figure 1

Specific growth rate (SGR, d^{-1}) of *R. subcapitata* exposed to two temperatures (24 °C and 29 °C) and increasing concentrations of a commercial sunscreen during 96 h. SSC1 (5 mg L^{-1}), SSC2 (10 mg L^{-1}), SSC3 (25 mg L^{-1}), SSC4 (50 mg L^{-1}), SSC5 (100 mg L^{-1}), and SSC6 (200 mg L^{-1}). Control (Ct) were not exposed to the sunscreen. Error bars indicate ± standard deviations, n=3. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$) for each temperature condition independently.

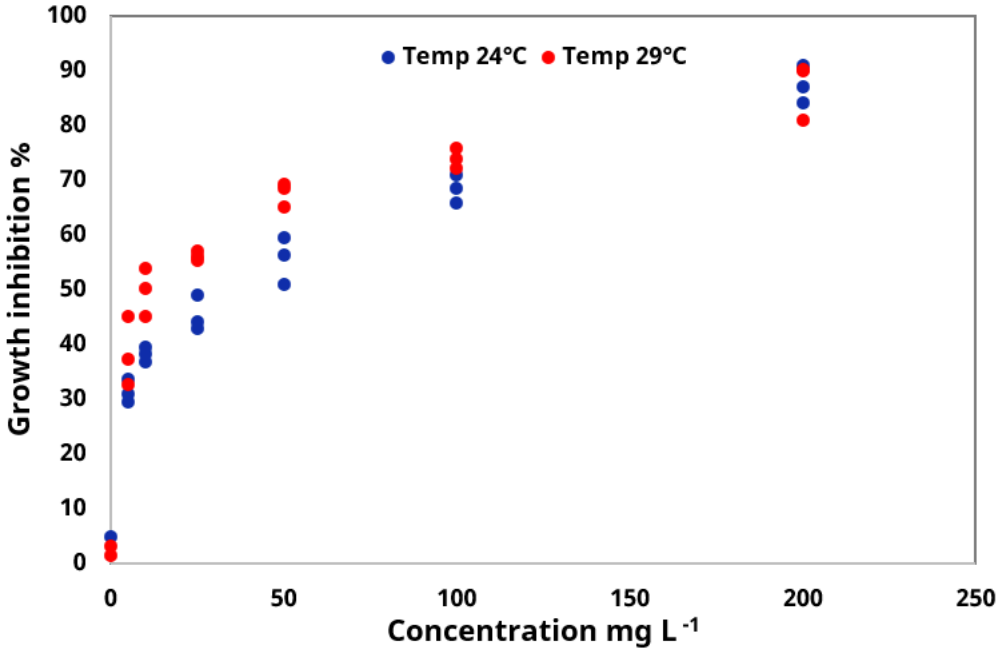


Figure 2

Growth inhibition of *R. subcapitata* exposure to two temperature levels (24 and 29 °C) and different concentrations of sunscreens (5,10, 25, 50, 100, and 200 mg L^{-1}) during 96 h.

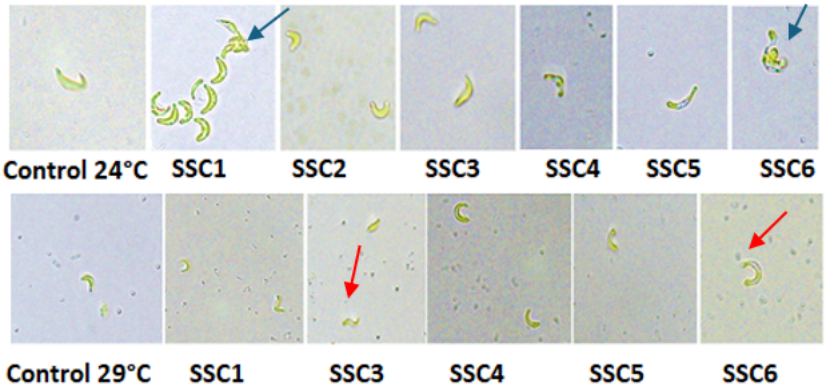


Figure 3

Effect of combined sunscreen concentration and temperatures (24°C and 29°C) on *R. subcapitata* at 96 h. **(A)** Optical microscopic images showing morphological changes in *R. subcapitata* under these treatments. SSC1 (5 mg L^{-1}), SSC2 (10 mg L^{-1}), SSC3 (25 mg L^{-1}), SSC4 (50 mg L^{-1}), SSC5 (100 mg L^{-1}), and SSC6 (200 mg L^{-1}). Control (Ct) was not exposed to the sunscreen. Blue arrows indicate cell aggregation; red arrows show cell deformation and elongation.

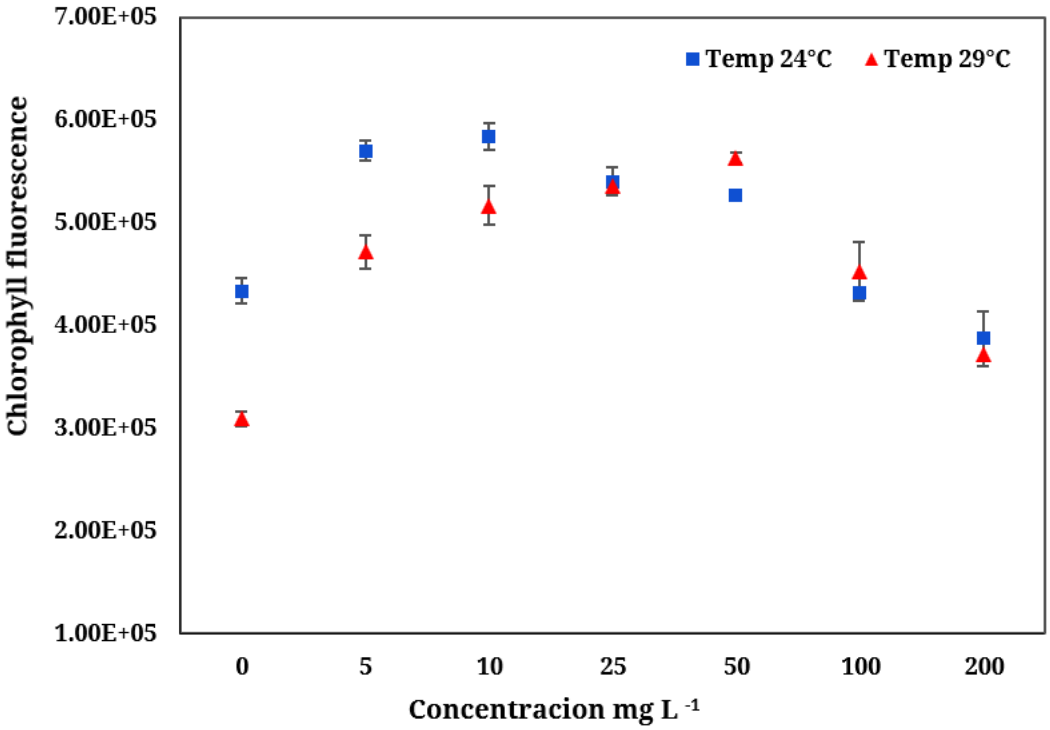


Figure 4

Chlorophyll a fluorescence in *R. subcapitata* under exposure to different concentrations of sunscreens and two temperature levels 24°C and 29°C. SSC1 (5 mg L^{-1}), SSC2 (10 mg L^{-1}), SSC3 (25 mg L^{-1}), SSC4 (50 mg L^{-1}), SSC5 (100 mg L^{-1}), and SSC6 (200 mg L^{-1}), Control (Ct).

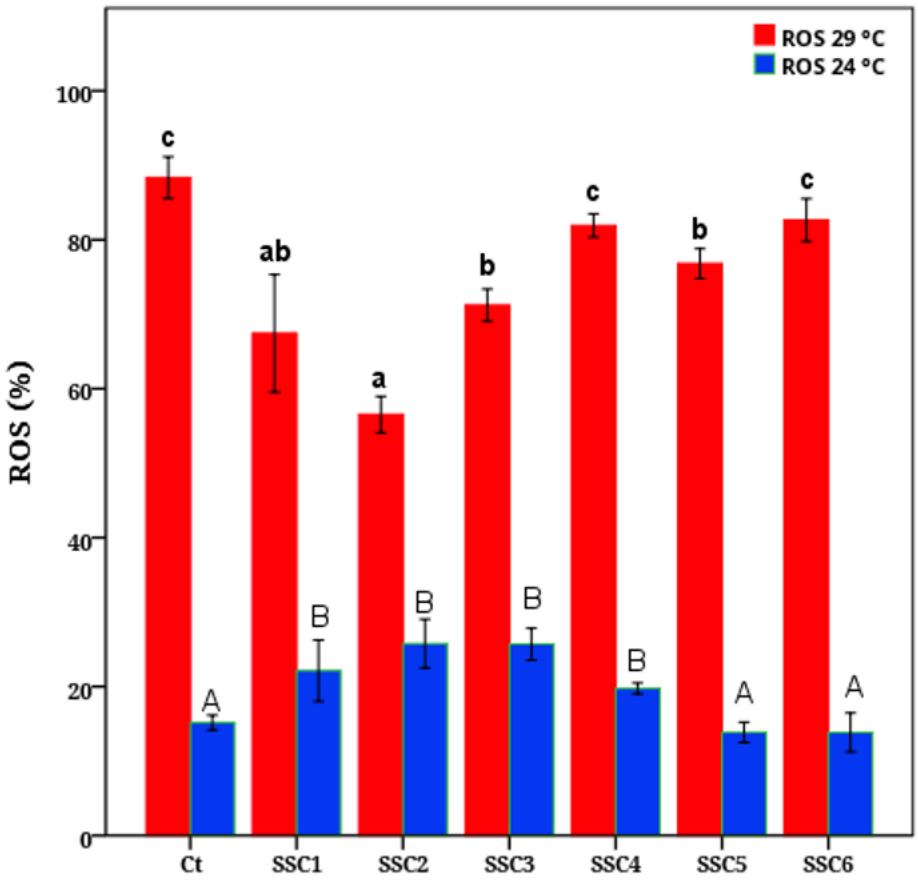


Figure 5

ROS generation under the combination of different concentrations of sunscreens and two temperature levels 24 and 29°C in *R. subcapitata*. SSC1 (5 mg L^{-1}), SSC2 (10 mg L^{-1}), SSC3 (25 mg L^{-1}), SSC4 (50 mg L^{-1}), SSC5 (100 mg L^{-1}), and SSC6 (200 mg L^{-1}), Control (Ct). Different lowercase letters indicate statistically significant differences ($p < 0.05$). Values are presented as means ± standard deviations (n = 3).

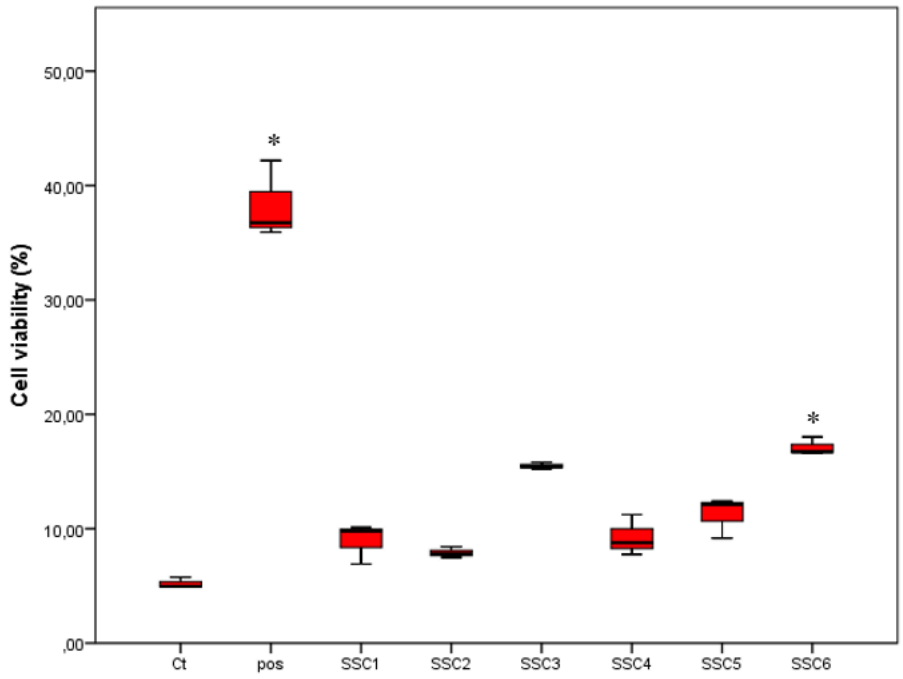


Figure 6

Percentage of cells with decreased viability at different concentrations of sunscreens and temperature level 29°C in green alga *R. subcapitata*. Values are mean ± SD (n = 3). SSC1 (5 mg L^{-1}), SSC2 (10 mg L^{-1}), SSC3 (25 mg L^{-1}), SSC4 (50 mg L^{-1}), SSC5 (100 mg L^{-1}), and SSC6 (200 mg L^{-1}), Control (Ct) and positive control (pos).(*)indicates statistically significant differences ($p < 0.05$).

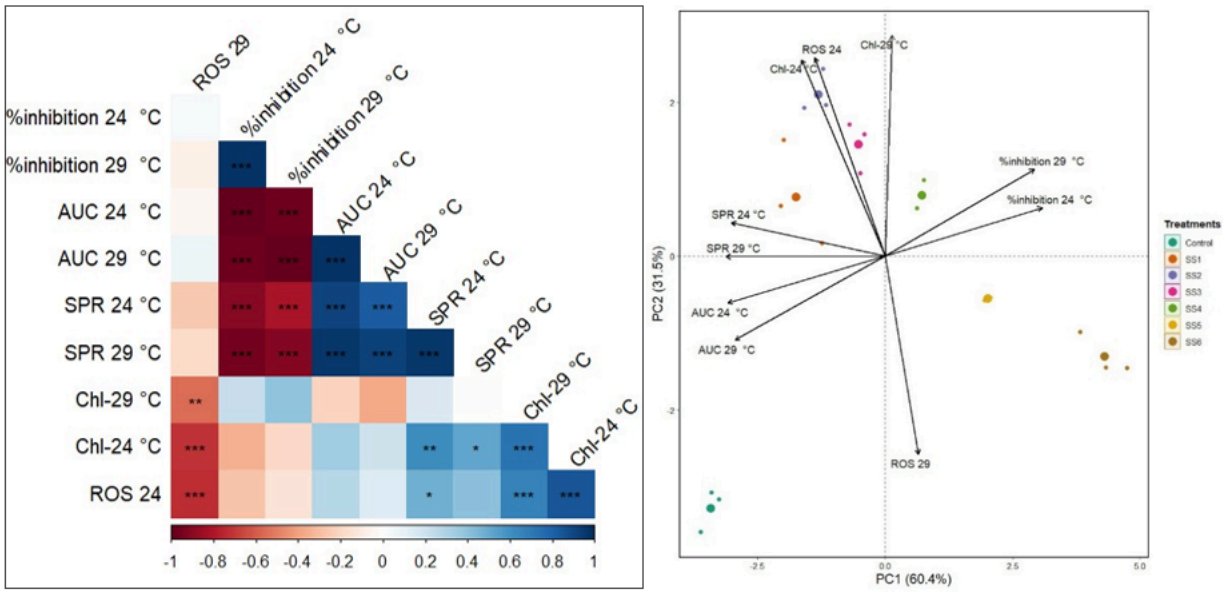


Figure 7

a. Pearson correlation matrix of physiological and biochemical parameters measured at 24 °C and 29 °C. Blue indicates positive correlations, red indicates negative correlations. Asterisks denote statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001).**b.** Principal Component Analysis (PCA) biplot showing distribution of treatments based on measured parameters; SSC1 (5 mg L^{-1}), SSC2 (10 mg L^{-1}), SSC3 (25 mg L^{-1}), SSC4 (50 mg L^{-1}), SSC5 (100 mg L^{-1}), and SSC6 (200 mg L^{-1}), Control (. Vectors represent loading scores of variables.

Supplementary Files

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