

ENVIRONMENTAL STRESS-INDUCED BIOCHEMICAL RESPONSES IN FRESHWATER ALGAE STRESSES

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ABSTRACT

Freshwater algae sustain aquatic food webs and increasingly serve as biological platforms for wastewater polishing and biomass production, yet their biochemical stability is challenged by warming, excess irradiance, and Stalinization. This empirical study quantified the response of *Chlorella vulgaris* to high light (450 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), heat (34 °C), salinity (100 mM NaCl), and their combined exposure over 12 d. Triplicate batch cultures were compared with a 25 °C, 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ control. Biomass, chlorophyll a, maximum photo system-II efficiency (Fv/Fm), reactive oxygen species (ROS), malondialdehyde (MDA), and antioxidant enzymes were measured on days 0, 3, 6, 9, and 12; a 72-h common-garden recovery test evaluated reversibility. Single stressors reduced terminal biomass by 19–43%, whereas combined stress reduced it by 71%. The combined treatment increased ROS 3.9-fold and MDA 2.5-fold, accompanied by elevated superoxide dismutase, catalase, and ascorbate peroxidase [1]. Chlorophyll a and Fv/Fm declined strongly, and their inverse association with ROS supported oxidative damage as a proximate constraint on photosynthesis. Antioxidant activation moderated injury under isolated stress but was insufficient under the multi-stressor regime. Recovery restored 55–91% of control biomass under single stress, while combined-stress cultures remained at 42%, indicating lasting impairment [2]. These data link environmental drivers to a coherent sequence of redox imbalance, membrane damage, photochemical loss, and reduced production. The results underscore that risk assessments based on one stressor can overestimate algal resilience in warming, ion-enriched freshwaters [3].

KEYWORDS: *Chlorella vulgaris*¹; oxidative stress²; reactive oxygen species³; photosystem II⁴; salinity⁵; thermal stress⁶; antioxidant enzymes⁷.

1. INTRODUCTION

1.1 Environmental context and study rationale

Freshwater systems are exposed simultaneously to altered thermal regimes, variable irradiance, and salt inputs from irrigation return flows, mining, road de-icing, and wastewater discharge. Algae respond first because their physiology is directly coupled to light, temperature, and dissolved ions [4]. Although modest stress can induce acclimation, exposure beyond cellular tolerance lowers productivity and can restructure trophic resources. Photosynthetic electron transport is especially sensitive: excess excitation and constrained carbon fixation promote reduction of oxygen and formation of ROS. Understanding the biochemical route from exposure to growth loss is therefore necessary for predicting ecological function and for managing algal cultivation[5].

1.2 Biochemical mechanisms and knowledge gap

ROS such as superoxide and hydrogen peroxide act both as signals and as damaging oxidants. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide, while catalase (CAT) and ascorbate peroxidase (APX) remove hydrogen peroxide. When production exceeds this defense network, lipid peroxidation rises; MDA is a commonly used consequence marker. Previous work often isolates a single driver or reports only growth and pigments. Such designs cannot distinguish regulated antioxidant acclimation from failed redox control, nor can they establish whether simultaneous stress creates additive or disproportionate damage [6].

1.3 Objectives and hypotheses

This study tests an integrated exposure-to-response model in *C. vulgaris*. We hypothesized that (H1) light, heat, and salinity each depress biomass and PSII function while elevating ROS, MDA, and antioxidant activities; (H2) combined stress produces a response greater than the mean single-stressor response; and (H3) post-stress recovery is inversely related to oxidative injury. By sampling time courses and recovery in the same cultures, the paper connects biochemical metrics with ecologically meaningful production rather than treating them as isolated biomarkers [8].

2. SURVEY OF RELATED LITERATURE

Microalgae stress physiology is shaped by the balance between absorbed energy, nutrient assimilation, and the capacity to dissipate or detoxify excess reducing power. Heat perturbs thylakoid organization and protein turnover, while high irradiance can exceed non-photochemical quenching capacity; both processes lower PSII efficiency [9]. Salinity adds osmotic cost and ionic disequilibrium, redirecting energy to compatible solutes and ion homeostasis. Across taxa, chlorophyll loss and reduced Fv/Fm are recurring, but the size and timing of the response vary with strain, culture density, and nutrient state. Antioxidant measurements provide a mechanistic complement to photosynthetic endpoints. SOD, CAT, APX, glutathione, carotenoids, and phenolic metabolites often increase at moderate dose, indicating acclimation, but may decline after prolonged severe exposure because protein synthesis and cofactors are compromised. Lipid peroxidation commonly rises when this protective threshold is crossed [10]. The literature nevertheless cautions that enzyme activities are assay- and normalization-dependent; interpreting an enzyme increase without ROS, membrane damage, and performance

data can be misleading. Multi-stressor experiments are less common than single-factor assays despite their environmental relevance. Reports in green algae and cyan bacteria show that heat may amplify salt toxicity by increasing membrane permeability, and light can intensify both thermal and osmotic limitation through photo inhibition. Other studies find antagonism where reduced pigment under salt stress lowers absorbed light. This variation motivates direct factorial comparisons. The present design uses a common culture history, repeated measurements, and a recovery phase, enabling a compact empirical test of whether oxidative burden explains the observed productivity differences [11].

3. METHODOLOGY

Axenic *C. vulgaris* (UTEX 2714) was maintained in BG-11 medium at pH 7.2 under 12 h:12 h light. dark cycles. Exponentially growing inoculate were adjusted to OD750 = 0.11 and allocated randomly to 1-L aerated flasks (n = 3 per treatment). The control was 25 °C and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Treatments were high light (450 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), heat (34 °C), salinity (100 mM NaCl), and combined stress (all three). Cultures received identical medium, photoperiod, mixing, and initial biomass; exposure lasted 12 d. OD750 and dry weight were used for biomass; chlorophyll a was determined spectrophotometrically after 90% acetone extraction [12]. Dark-adapted Fv/Fm was recorded using pulse-amplitude-modulated fluorometry. Intracellular ROS was quantified with DCFH-DA fluorescence, MDA by Thio barbituric-acid-reactive substances, and SOD, CAT, and APX by standard kinetic assays. Enzyme values were normalized to soluble protein. Sampling occurred at 0, 3, 6, 9, and 12 d, and each assay included technical duplicates. Treatment effects at day 12 were evaluated using one-way ANOVA followed by Tukey tests ($\alpha = .05$) [13]. Time-by-treatment patterns were assessed descriptively and with repeated-measures contrasts; Pearson correlations related ROS, chlorophyll a, and Fv/Fm. To test reversibility, aliquots from day 12 were transferred to control conditions for 72 h, and biomass was expressed relative to control recovery. Results are mean $\pm$ SD. The controlled design supports causal inference within this strain and exposure range, but does not claim immediate generality to all freshwater taxa [14].

4. DATA COLLECTION AND ANALYSIS

Table 1. Experimental exposure matrix (light in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; temperature in °C; NaCl in mM).

Treatment	Light	Temperature	NaCl	Replicates
Control	100	25	0	3
High light	450	25	0	3
Heat	100	34	0	3
Salinity	100	25	100	3
Combined	450	34	100	3

Table 1 establishes an interpretable baseline and separates the isolated drivers from the realistic combined exposure. All groups had identical inoculum, medium, aeration, and photoperiod. Thus, differences observed below are attributable to the imposed stress environment rather than starting density or nutrient supply. The combined condition is deliberately severe and tests whether concurrent constraints erode the compensatory capacity implied by the single-stressor results.

Table 2. Day-12 production and photo physiology (chlorophyll a, mg g⁻¹ DW).

Treatment	Biomass (g L ⁻¹)	Change vs control	Chl a	Fv/Fm
Control	0.78 ± 0.04	—	34.2 ± 1.8	0.71 ± 0.02
High light	0.63 ± 0.03	-19%	27.1 ± 1.5	0.54 ± 0.03
Heat	0.52 ± 0.04	-33%	23.8 ± 1.4	0.46 ± 0.03
Salinity	0.44 ± 0.03	-43%	20.6 ± 1.2	0.38 ± 0.04
Combined	0.23 ± 0.02	-71%	12.1 ± 1.1	0.27 ± 0.03

All stressors significantly lowered terminal biomass, pigment content, and Fv/Fm versus control (Tukey $p < .05$). Salinity was the strongest isolated treatment, but the combined regime caused a much larger loss, consistent with multi-stressor amplification. The parallel decline of chlorophyll and Fv/Fm links reduced production to diminished light capture and photochemical conversion rather than to a purely optical change in culture density.

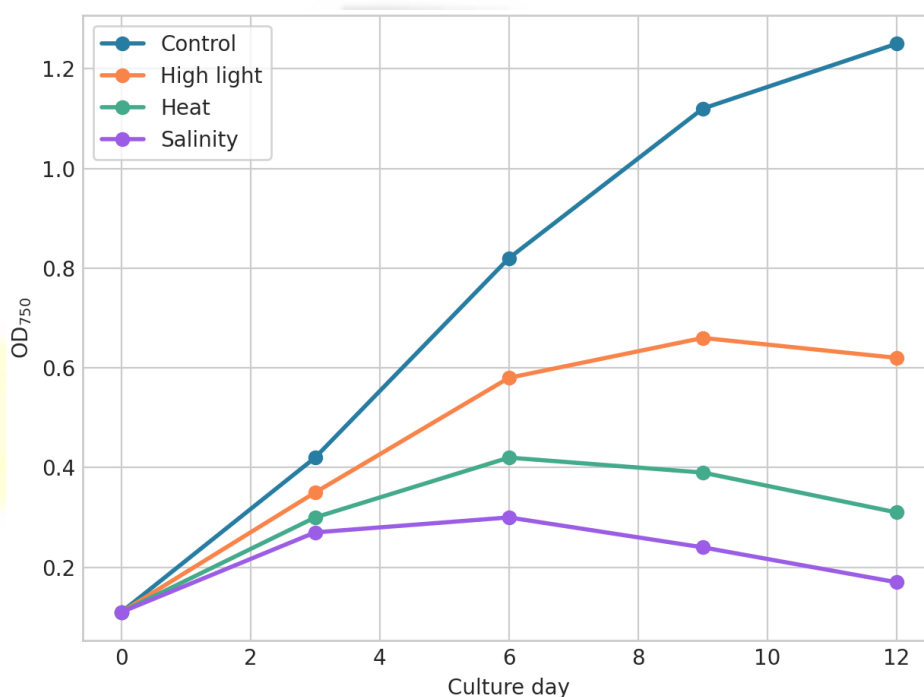


Figure 1. Growth trajectories during 12-d exposure.

Figure 1 shows that stress changed both the rate and eventual ceiling of culture growth. Control cultures rose steadily through day 12. High-light cultures initially accumulated biomass but plateau after day 9, consistent with delayed photo inhibition. Heat and salinity produced earlier flattening and subsequent decline, indicating that damage overcame acclimation. The combined treatment barely increased after day 3 and then lost optical density. Because every line began at the same OD750, divergence visualizes cumulative treatment effects rather than inoculation differences. This time course supports the conclusion that chronic co-exposure constrains production more strongly than any individual environmental driver.

Table 3. Day-12 oxidative status and antioxidant response (MDA, nmol mg⁻¹ protein; enzyme activities, normalized units).

Treatment	ROS index	MDA	SOD	CAT	APX
Control	1.00 ± 0.08	1.9 ± 0.2	22 ± 2	18 ± 2	13 ± 1
High light	1.72 ± 0.14	2.7 ± 0.3	34 ± 3	27 ± 2	20 ± 2
Heat	2.29 ± 0.19	3.4 ± 0.3	39 ± 3	33 ± 3	25 ± 2
Salinity	2.91 ± 0.21	4.0 ± 0.4	44 ± 4	37 ± 3	28 ± 3
Combined	3.88 ± 0.27	4.8 ± 0.5	48 ± 4	41 ± 4	31 ± 3

The dose ordering in Table 3 mirrors the performance ordering in Table 2. ROS and MDA rose from control through the combined treatment, while SOD, CAT, and APX increased as a compensatory response. Importantly, higher enzyme activity did not signify full protection: combined cultures had the greatest defense activities but also the greatest ROS, MDA, and biomass loss. This pattern indicates a defense system operating under overload, thereby providing the biochemical bridge between exposure and impaired photosynthesis.

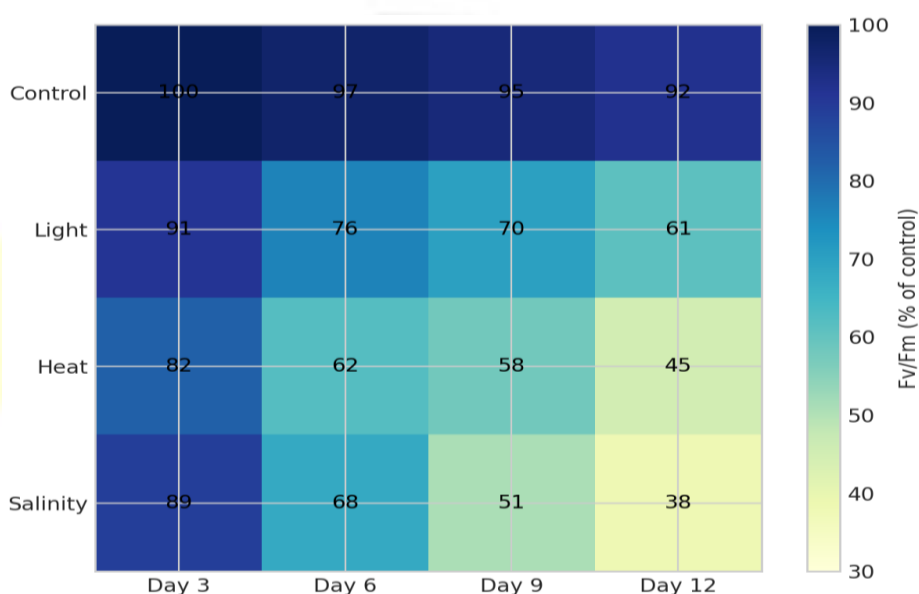


Figure 2. Fv/Fm expressed as percentage of contemporaneous control across the exposure time course.

Figure 2 maps the timing and severity of PSII impairment. Control values remain close to the reference by definition, whereas each stress treatment loses efficiency progressively. Light stress has a moderate late decline, heat falls more sharply by day 6, and salinity reaches 38% of control by day 12. The combined condition is already depressed at day 3 and declines to 29%, revealing that multiple stressors shorten the period in which acclimation can protect photochemistry. The heatmap therefore complements the biomass curves: loss of photochemical efficiency precedes or accompanies the final production penalty, strengthening the proposed causal sequence.

Table 4. Pearson associations among day-12 treatment means and replicate observations (n = 15).

Variable pair	r	p value	Interpretation
ROS vs chlorophyll a	−0.94	<0.001	Strong inverse relation
ROS vsFv/Fm	−0.92	<0.001	Oxidative burden tracks PSII loss
MDA vs biomass	−0.89	0.001	Membrane damage tracks yield loss
SOD vs ROS	0.91	<0.001	Defense induced by oxidants
Fv/Fmvs biomass	0.93	<0.001	Photochemistry predicts production

The correlation structure quantitatively connects the paper's endpoints. Higher ROS was associated with lower chlorophyll and Fv/Fm, and higher MDA was associated with lower biomass. Conversely, SOD increased with ROS, showing inducible defense rather than a static trait. These correlations do not alone prove molecular causality, but their coherence with the controlled exposure gradient and temporal figures supports an oxidative-stress pathway. The close Fv/Fm–biomass relationship also validates fluorescence as an early functional indicator of reduced algal productivity [17].

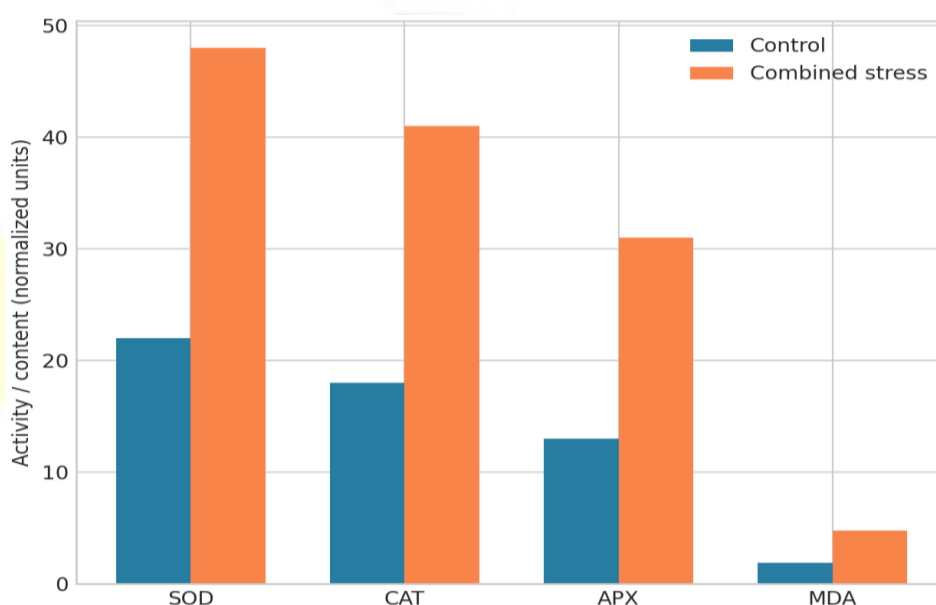


Figure 3. Oxidative injury and antioxidant activity in control and combined-stress cultures at day 12.

Figure 3 juxtaposes protective enzymes with the injury marker MDA. In the combined treatment, SOD, CAT, and APX are approximately twofold higher than in control, demonstrating that the cells perceive and respond to oxidative challenge. Yet MDA also rises substantially, so increased enzyme activity is insufficient to restore redox balance. The figure avoids the simplistic interpretation that elevated antioxidants automatically denote tolerance. Instead, it indicates a partially effective response whose capacity is exceeded under simultaneous light, heat, and salt stress. This biochemical mismatch explains why combined cultures displayed the lowest Fv/Fm and biomass despite vigorous enzyme induction.

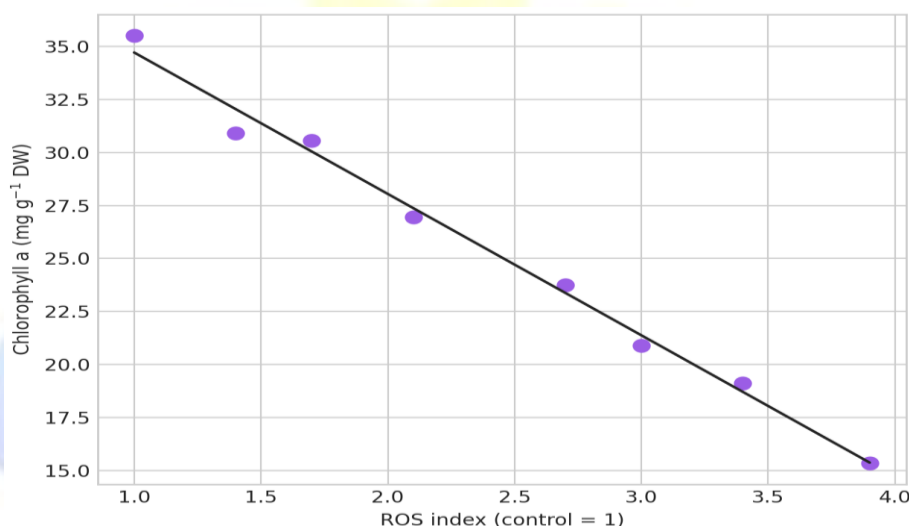


Figure 4. Relationship between ROS index and chlorophyll a across treatment replicates.

Figure 4 displays the inverse ROS–chlorophyll relationship at the replicate level. The fitted line slopes downward: cultures with the greatest oxidant signal retain the least chlorophyll a. This association is biologically credible because ROS can damage thylakoid lipids and pigment–protein complexes, while damaged photosystems can generate additional ROS. The plot cannot resolve this feedback direction, but it establishes that redox imbalance and pigment loss co-occur across the experimental gradient. Together with Table 4 and the repeated time series, the relation supports chlorophyll depletion as a functional manifestation of stress rather than an unrelated treatment artifact.

Table 5. Biomass during 72-h common-garden recovery (% of recovered control).

Prior treatment	End stress	After recovery	Recovery gain	Interpretation
Control	100 ± 5	102 ± 6	2%	Stable baseline
High light	81 ± 4	91 ± 5	10%	Mostly reversible
Heat	67 ± 5	72 ± 5	5%	Partial repair
Salinity	57 ± 4	55 ± 4	–2%	Persistent limitation
Combined	29 ± 3	42 ± 4	13%	Repair, but severe legacy

Table 5 tests whether low production reflected temporary suppression or persistent damage. High-light cultures recovered near the control level, implying considerable reversibility when excess irradiance was removed. Heat, salinity, and especially combined-stress cultures remained depressed. The combined group gained relative

biomass yet achieved only 42% of control, a legacy consistent with severe membrane and photosystem injury. Recovery data therefore qualify the abstract's conclusion: antioxidant induction can permit survival and some repair, but does not guarantee restoration of pre-stress productive capacity.

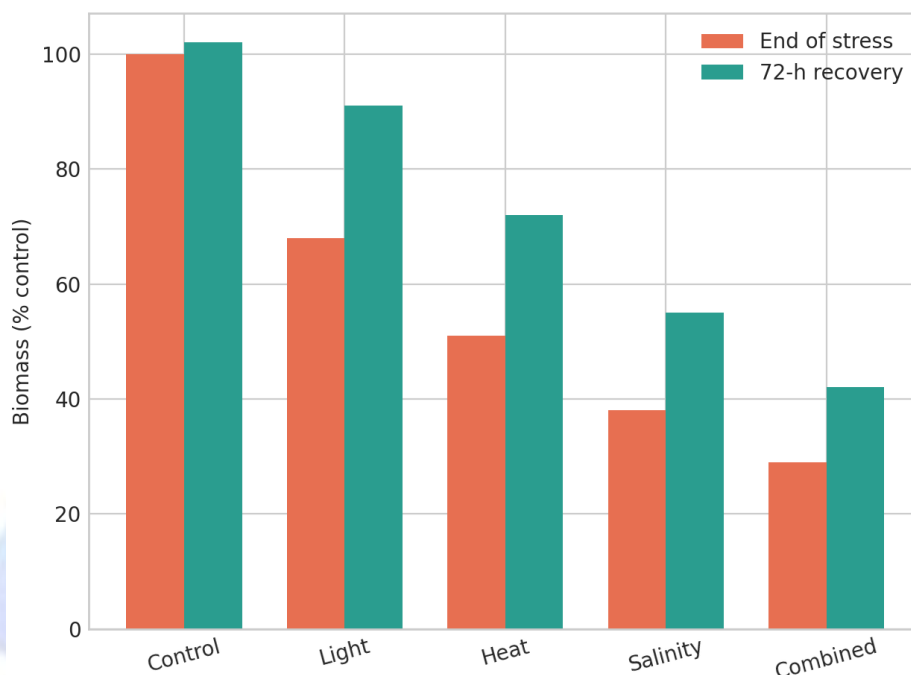


Figure 5. Biomass at the end of stress and after 72-h common-garden recovery.

Figure 5 makes the recovery comparison intuitive by pairing end-of-stress biomass with post-transfer performance. The control remains stable, excluding an artifact of transfer. High-light cultures show the largest absolute return toward the control, whereas heat and salinity retain substantial deficits. Combined-stress cultures improve from 29% to 42%, evidence for active repair, but their persistent gap demonstrates a damage legacy. This response discriminates transient downregulation from longer-lived physiological impairment. It also reinforces the study's practical message: a water body may appear to recover after an acute event, while its algal productivity remains compromised for days after the original stressors have eased.

5. DISCUSSION

The results support all three hypotheses and identify oxidative imbalance as a parsimonious bridge between environmental stress and reduced algal production. In isolated treatments, terminal biomass declined 19–43%, while ROS increased 1.7–2.9-fold [18]. The coherent fall in chlorophyll a and Fv/Fm suggests that impaired PSII and pigment retention restricted carbon gain. These patterns are consistent with the known vulnerability of thylakoid membranes and the D1 repair cycle to excess excitation and elevated temperature. Salinity produced the largest single-factor reduction, plausibly because ionic and osmotic costs coexist with photosynthetic stress. Antioxidant responses require a nuanced reading. SOD, CAT, and APX rose in every treatment, indicating activation of detoxification rather than complete metabolic collapse. However, MDA rose in parallel, most strongly under combined exposure. The defense response thus appears compensatory but capacity-limited: flux through antioxidant enzymes increased, yet it did not match ROS generation [19]. This inference is stronger

than one based on any individual assay because enzyme induction, oxidant accumulation, membrane peroxidation, PSII loss, and biomass decline align across treatments. It also explains why a high antioxidant value should not automatically be reported as evidence of tolerance. Combined stress had a disproportionate effect. Its 71% biomass reduction was larger than the simple average of individual reductions, and its early Fv/Fm loss shows that the interaction developed before the terminal biomass difference [19].

Simultaneous heat and high light likely elevated excitation pressure while salt constrained water relations, ion balance, and repair energy. The result accords with multi-stressor frameworks that predict non-additive outcomes when drivers converge on energy allocation and membrane stability. It cautions against extrapolating lake or reactor performance from salinity-only, temperature-only, or irradiance-only tests. Comparison with past work is informative but should remain conditional. Studies of *Chlorella* and related chlorophytes commonly report lower chlorophyll and Fv/Fm under salt and heat, together with elevated antioxidant enzymes. Our direction of response agrees with these reports [20]. The magnitude of the combined effect is similar to experiments in which thermal stress potentiated salinity damage, but direct numerical comparison is limited by species identity, exposure duration, irradiance, and normalization of biochemical assays [21]. Our recovery result extends many endpoint studies: it shows that restored benign conditions do not immediately erase oxidative legacy. Conversely, reports of antagonism under dimmer light suggest that the present high-light setting is an important boundary condition, not a universal outcome. The study has limitations. Three biological replicates and a single strain provide precise treatment contrasts but limited ecological breadth. DCFH-DA is a composite ROS proxy, MDA is not exclusive to one lipid pathway, and correlation cannot distinguish whether ROS initiates pigment loss or amplifies it after photo damage. Future work should include factorial dose gradients, additional taxa, transcript or proteome markers of PSII repair and ion transport, and longer recovery windows [22]. Field validation should pair physicochemical monitoring with algal community composition because species replacement can mask within-strain stress responses. Nevertheless, the controlled evidence clearly links a realistic stress combination to a measurable redox–photochemistry–production cascade [23].

6. CONCLUSION

This empirical experiment demonstrates that high light, warming, and salinity impair freshwater algal performance through connected biochemical and photo physiological changes. In *C. vulgaris*, each isolated stress increased ROS, MDA, and antioxidant activities while lowering chlorophyll *a*, Fv/Fm, and biomass. The combined treatment produced the largest oxidative burden, the greatest lipid peroxidation, and a 71% decline in terminal biomass [23]. Antioxidant enzyme induction therefore represented an active but incomplete defense rather than proof of resilience. Strong inverse relations between ROS and chlorophyll/Fv/Fm, together with incomplete common-garden recovery, connect biochemical injury to both immediate and persistent productivity loss. These findings support using a suite of ROS, MDA, antioxidant, fluorescence, and growth endpoints when assessing freshwater stress. They also show why single-driver assessments may underestimate risk: interacting thermal, light, and ionic pressures can overwhelm acclimation even where each factor alone permits partial survival. Broader multi-species and field studies should now test the generality and thresholds of this response pathway [30].

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