Sustainable Aquatic Research (2025) 4(3):270-280
https://doi.org/10.5281/zenodo.18007327
e-ISSN: 2822-4140

SUSTAINABLE AQUATIC RESEARCH
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RESEARCH PAPER

Oxidative Stress and Antioxidant Response of *Chlorella vulgaris* Under Chlorpyrifos Exposure

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Citation

Serdar, O, Aydın, A. N., Ölçülü, A., Çimen, Ç. C. I., Ak. P. T., Derman, T., Pala, A., Yıldırım, C. N., (2025). Oxidative stress and antioxidant response of *Chlorella vulgaris* under chlorpyrifos exposure. *Sustainable Aquatic Research*, *4*(3), 270-280. https://doi.org/10.5281/zenodo.18007327

Article History

Received: 07 November 2025
Received in revised form:11 December 2025
Accepted: 12 December 2025
Available online: 24 December 2025

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Keywords

Chlorella vulgaris
Chlorpyrifos
Oxidative stress
Antioxidant enzymes
Aquatic toxicity
Biomarkers

Handling Editor

Erklan Can

Abstract

Chlorpyrifos (CPF), a widely used organophosphate pesticide, frequently enters aquatic environments and poses potential risks to non-target primary producers such as microalgae. This study investigated the oxidative stress response of *Chlorella vulgaris* exposed to sublethal concentrations of CPF based on the 96-h EC_{50} value (4.86 mg/L). Experimental groups were designed using 1/8, 1/4, and 1/2 EC_{50} concentrations, and biochemical responses were evaluated at 24, 48, 72, 96, and 120 hours. Results demonstrated that CPF exposure induced significant changes in antioxidant enzyme activities. Superoxide dismutase (SOD) and catalase (CAT) activities increased at specific exposure periods, while glutathione peroxidase (GPx) exhibited concentration- and time-dependent alterations. Glutathione (GSH) levels increased throughout exposure and during the elimination phase, indicating activation of non-enzymatic antioxidant defense. In contrast, thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation, showed no statistically significant change.

Introduction

Pesticides used in the construction of large industrial facilities, the release of waste into the environment, its mixing with water, and increased agricultural productivity are just a few of the factors contributing to environmental pollution (Aydın et al., 2022). Chlorpyrifos (O, O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) is one of the common insecticides used in agricultural and urban applications and is effective in controlling plant and soil insects in various food crops (Schiff et al., 2002). Although chlorpyrifos is among the banned pesticides worldwide and in Türkiye, it continues to be used in many major countries, such as the United States.

Pesticides, such as CPF, are chemicals that affect, alter, or inhibit various biological processes. These toxic substances can reach aquatic environments through direct spraying or runoff from surrounding terrestrial areas. In recent years, agrochemical applications on economically important crops have increased, making them one of the most common organic pollutants in aquatic ecosystems (Prado et al., 2009).

Microalgae generally produce antioxidant reactions through enzymes against excessive ROS due to toxic substances, the most important of these enzymes are superoxide dismutase (SOD), catalase (CAT) and GPx (Blokhina et al., 2006), TBARS and GSH are indicators that reflect the organism's cell damage and response to adverse environment (Mu et al., 2017).

Microalgae play a crucial role in aquatic ecosystems, being a key component of aquatic food chains due to their fundamental role in energy conversion and maintenance of ecosystem food webs. Because microalgae are at the base of aquatic food webs: Chlorpyrifos pollution can reduce algal biomass affects oxygen production ınfluences zooplankton populations Impacts overall aquatic ecosystem health. Their short generation time and rapid response to environmental changes are important for microalgae to assess toxic and genotoxic effects at the cellular level and to conduct early assessment studies (Villem, 2011; Martinez et al., 2015).

The limited number of studies on this topic in the literature makes it even more important to investigate the oxidative stress responses of *Chlorella vulgaris* under chlorpyrifos exposure. Such an investigation not only reveals the biochemical and physiological alterations occurring in algal cells such as the production of reactive oxygen species, fluctuations in antioxidant enzyme activities, and disruption of cell membrane integrity but also contributes to understanding the cascading ecological effects that may arise from reductions in algal biomass.

The potential inhibitory effects of chlorpyrifos on photosynthetic pigments may lead to a marked decrease in oxygen production, thereby weakening primary productivity in freshwater ecosystems. This decline in primary production can, in turn, reduce the availability of food resources for organisms at lower trophic levels, such as zooplankton. Consequently, pesticide exposure may generate indirect ecological impacts extending from algae to zooplankton communities and even to higher trophic levels.

This comprehensive evaluation is critically important for the sustainable management of freshwater ecosystems. Considering the increasing anthropogenic agricultural pollution, understanding the responses of algae to pesticide exposure is essential for maintaining ecosystem health, identifying potential bioindicator species, and predicting ecological risks.

In this study, *C. vulgaris* was determined as a model organism to investigate the effects of CPF on non-target organisms and it was aimed to determine the changes in SOD, CAT and GPx activities and TBAS and GSH levels caused by CPF in *C. vulgaris*

Materials and Methods

Chemicals

Analytical-grade chlorpyrifos (CPF; O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) was obtained as a high-purity commercial formulation. A stock solution was prepared in distilled water and stored at 4 °C in the dark until use. Working concentrations were prepared freshly for each experiment by diluting the stock solution with culture medium.

Preparation of Chlorella vulgaris Culture

Microalgae Culture Preparation

Pure cultures of *Chlorella vulgaris* (UTEX, University of Texas Algal Culture Collection) were maintained in the Aquatic Sciences Laboratory of Munzur University. The starter culture ($3.5–4\times10^6$ cells/mL) was grown in sterilized proteose peptone medium under controlled laboratory conditions:

- **Temperature:** 20 ± 1 °C
- **Photoperiod:** 12:12 h light–dark cycle
- **Light intensity:** 3200 lux
- **Agitation:** orbital shaker, gentle daily mixing

Culture vessels (19-L demijohns) were washed with hot water, treated with hydrochloric acid (HCl), rinsed thoroughly, and filled with UV-sterilized freshwater. To ensure sterilization, 10 mL sodium hypochlorite was added to each demijohn and aerated for 5 min, followed by a 24-h waiting period. Residual chlorine was neutralized by adding 3 g sodium thiosulfate and aerating for 45 min.

The medium was enriched with commercial fertilizer (20:20:20 N:P:K + trace elements, 40 mg/L). Approximately 250 mL of the starter culture was inoculated into each demijohn. Cultures were grown until reaching the logarithmic phase ($1–2\times10^6$ cells/mL), after which they were harvested for toxicity tests.

Acute Toxicity Test (EC_{50} Determination)

Acute toxicity of CPF to *C. vulgaris* was assessed following OECD Test Guideline 201 (2011). Tests were conducted in sterile 100-mL plastic containers containing 45 mL CPF solution and 5 mL microalgal culture (final volume: 50 mL). Exposure periods were 24, 48, 72, and 96 h.

At each time point, 1 mL of culture was sampled and stained with 0.1 mL trypan blue to distinguish viable and non-viable cells. Samples were incubated for 10 min in the dark and counted using a Neubauer hemocytometer under a light microscope. Each concentration was tested with three replicates.

The 96-h EC_{50} value was calculated via probit analysis, based on three independent acute toxicity trials (Yoon et al., 2007; Erdem et al., 2014; Özkaleli and Erdem, 2017).

Sublethal Exposure Experiments

Sublethal concentrations were selected as fractions of the determined EC_{50} value (4.86 mg/L):

- Control: 0 mg/L CPF
- Low concentration: 1/8 EC_{50}
- Medium concentration: 1/4 EC_{50}
- High concentration: 1/2 EC_{50}

Microalgae were exposed for up to 96 h, and samples were collected at 24, 48, 72, and 96 h. Following the exposure period, cultures were transferred to CPF-free medium to assess elimination, and additional samples were collected after 24 h.

All samples were stored at −86 °C until biochemical analysis

Table 1. EC_{50} values calculated by probit analysis of CPF pesticide active ingredient on *C. vulgaris.*

C. vulgaris	EC_{50} mg/L
Recurrence 1	5.22
Recurrence 2	4.58
Recurrence 3	4.78
Average value	4.86
Standard deviation	0.33

Trial Design and Application Concentrations

The EC_{50} values of the CPF for microalgae were determined based on the acute (EC_{50}) tests. Based on the EC_{50} values of the CPF pollutants, the following were determined:

Group A: Unexposed to any pollutant (control);

Group B: Pollutant at 1/8 the EC_{50} value of the pollutant;

Group C: Pollutant at 1/4 the EC_{50} value of the pollutant;

Group D: Pollutant at 1/2 the EC_{50} value of the pollutant.

Trial groups were formed, and the trials were conducted with three replicates.

Biochemical Analyses

For antioxidant enzyme analyses, 0.5 g algal pellet from each sample was homogenized in phosphate-buffered saline (PBS; pH 7.4; 1:10 w/v) on ice. Homogenates were centrifuged at 17,000 rpm for 15 min at 4 °C, and supernatants were collected for analysis.

The following biomarkers were measured using ELISA microplate readers and CAYMAN brand commercial test kits:

- Superoxide dismutase (SOD) activity
- Catalase (CAT) activity
- Glutathione peroxidase (GPx) activity
- Reduced glutathione (GSH) levels

For lipid peroxidation analysis, samples were homogenized in 1.15% KCl (1:10 w/v) and centrifuged at 3500 rpm for 15 min. Thiobarbituric acid reactive substances (TBARS) levels were determined using standard spectrophotometric methods.

Statistical Analysis

EC_{50} values were calculated using probit analysis (SPSS 24.0). Biochemical data were analyzed via one-way ANOVA followed by Duncan's multiple range test ($p<0.05$) to compare differences among treatment groups. Temporal variations were assessed using two-way ANOVA and independent t-tests where appropriate. All results are presented as mean ± standard deviation (Aydın et al. 2022).

Results

SOD Activity

The changes in SOD enzyme activity in *C. vulgaris* exposed to CPF are shown in Figure 1. Increases occurred in the CPF-exposed groups compared to the control at 24 and 48 hours, while changes in the other groups were not statistically significant ($p>0.05$).

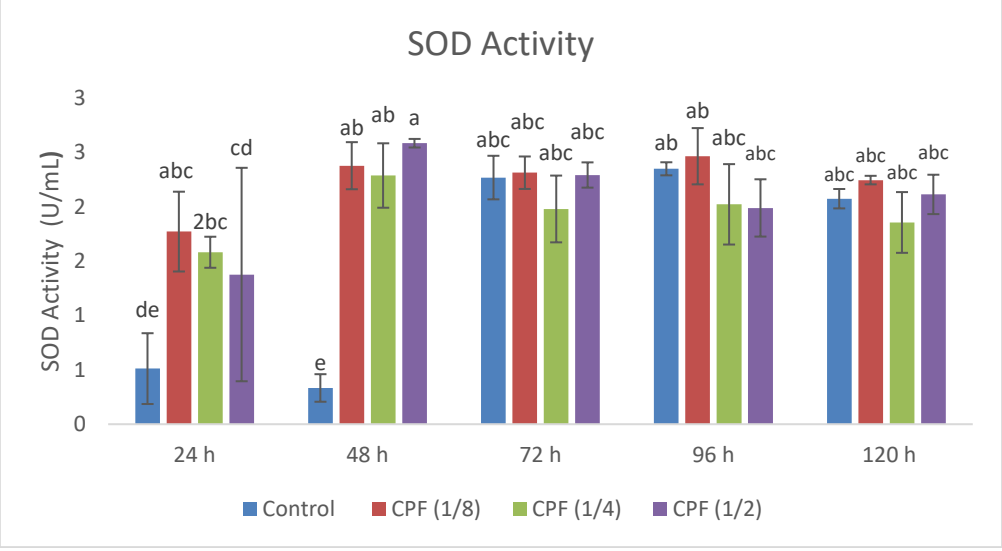

Figure 1. SOD activity after CPF exposure on *C. vulgaris*
There are differences at $p<0.05$ level between the data shown with different letters on the column within the same time group.

CAT Activity

Changes in CAT activity following CPF application in *C. vulgaris* are shown in Figure 2. Statistically significant ($p<0.05$) increases were observed compared to the control group in all groups except the 72-hour period. The decrease in elimination time at 120 hours was found to be statistically significant.

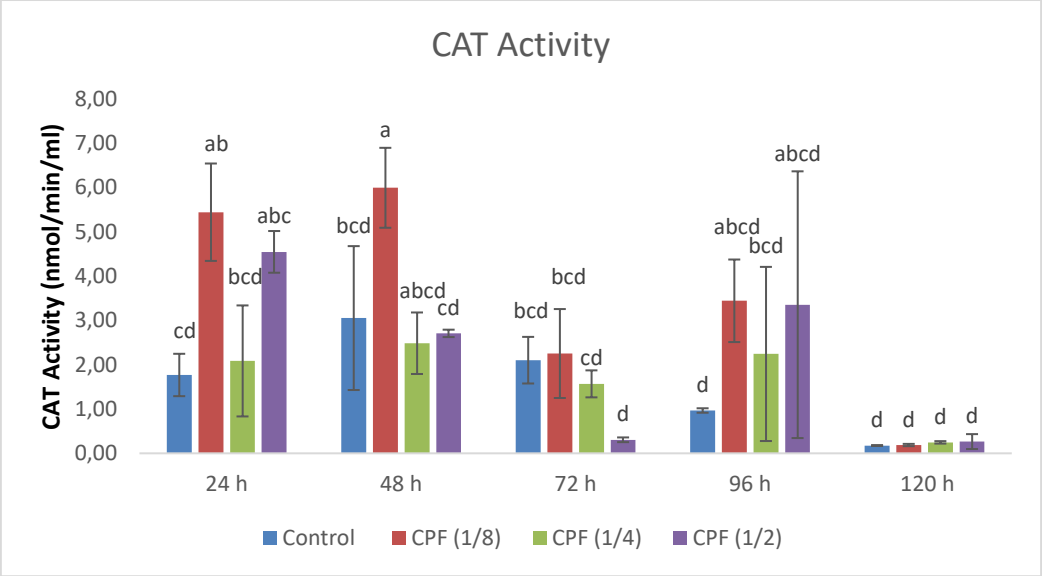

Figure 2. CAT activity after CPF on *C. vulgaris*
There are differences at $p<0.05$ level between the data shown with different letters on the column within the same time group.

GPx Activity

Changes in GPx activity following CPF application in *C. vulgaris* are shown in Figure 3. Statistically significant differences were found between the control group and the applied concentrations under CPF exposure ($p<0.05$). Increases in GPx activity were observed during the elimination period.

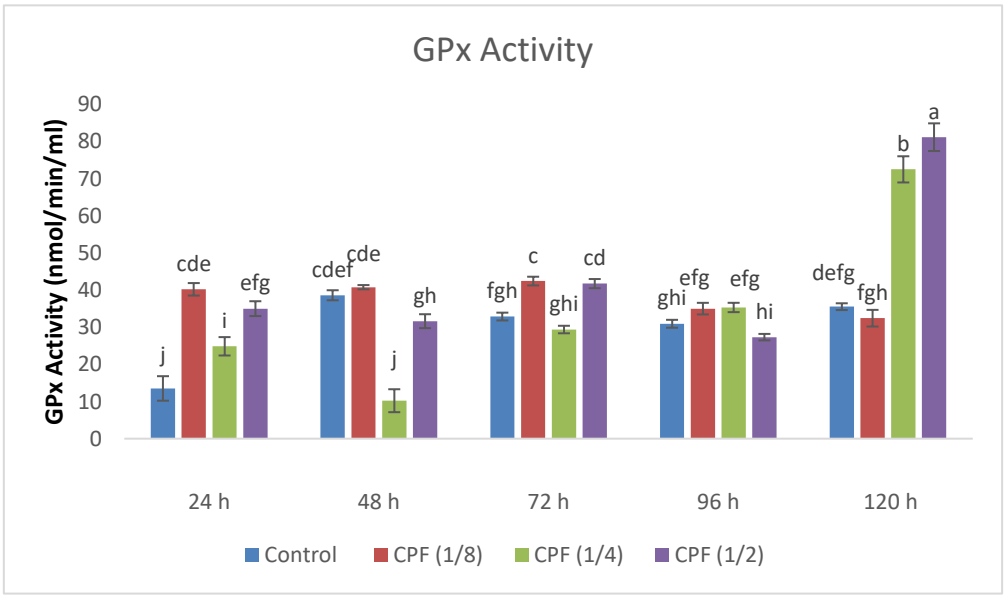

Figure 3. GPx activity after CPF exposure on *C. vulgaris*
There are differences at $p<0.05$ level between the data shown with different letters on the column within the same time group.

TBARS Level

The change in TBARS levels in *C. vulgaris* exposed to CPF is shown in Figure 4. Although TBARS levels increased in the CPF-exposed groups compared to the control group, no statistically significant difference was found ($p>0.05$). The change in elimination time was not statistically significant.

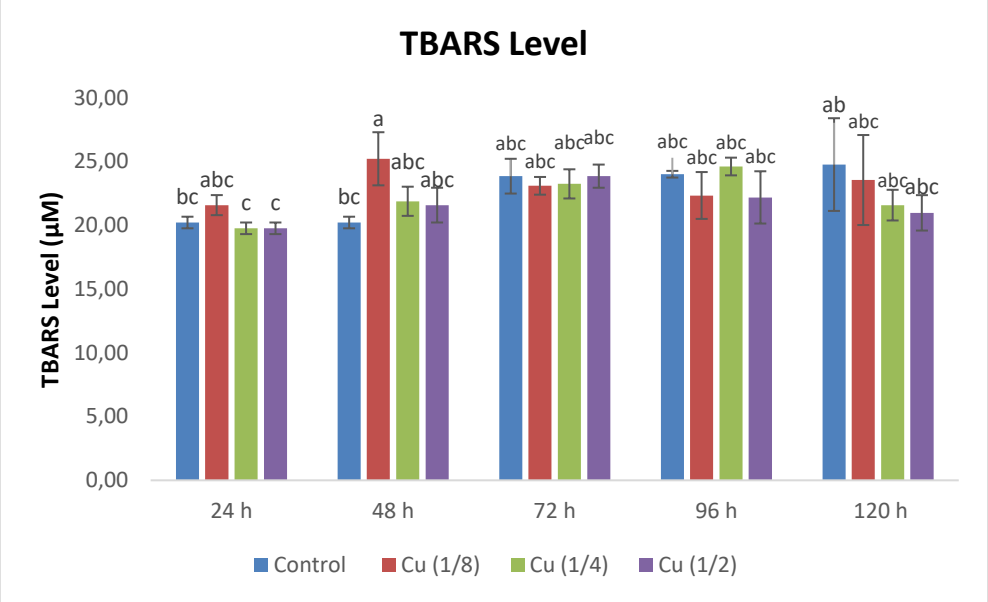

Figure 4. TBARS level after CPF exposure on *C. vulgaris*
There are differences at $p<0.05$ level between the data shown with different letters on the column within the same time group.

GSH Level

The change in GSH levels in *C. vulgaris* exposed to CPF is shown in Figure 5. A statistically significant difference was found between the CPF-exposed and control group samples in terms of GSH levels and elimination time ($p<0.05$).

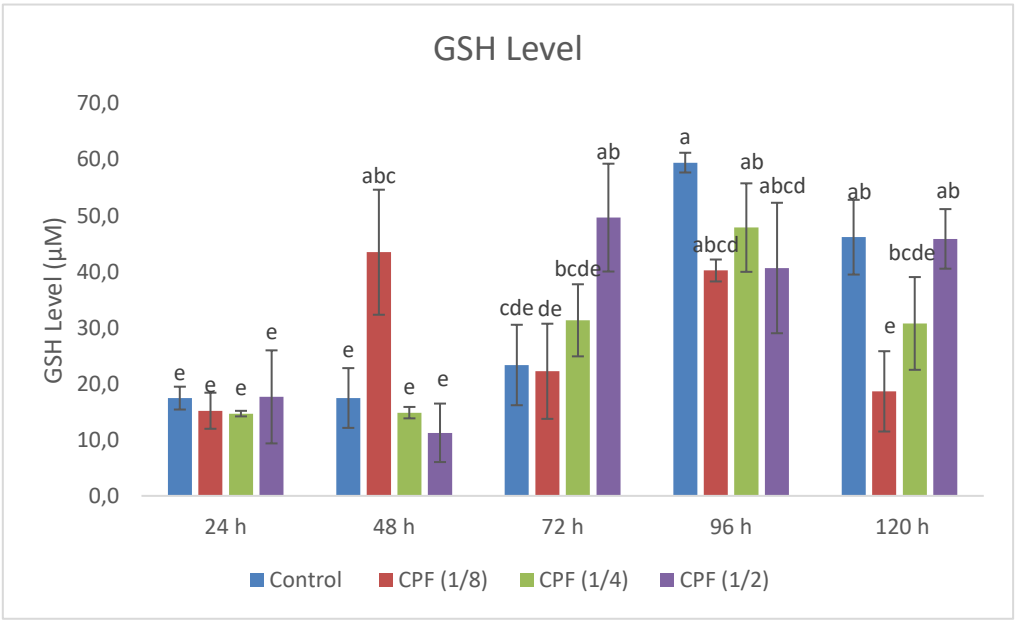

Figure 5. GSH Level after CPF exposure on *C. vulgaris*
There are differences at $p<0.05$ level between the data shown with different letters on the column within the same time group.

Discussion

The use of pesticides against pests in agricultural areas and homes has become widespread. Pesticides have the ability to bioaccumulate and concentrate in the bodies of living organisms (Braune et al., 2005). Pesticides can be transported to aquatic ecosystems through various means, including spraying, atmospheric transport, agricultural runoff, misuse, and improper disposal (Singh and Singh, 2008). Aquatic ecosystems are highly vulnerable to pesticide pollution, which negatively impacts aquatic life (Narra et al., 2015). Pesticides pose significant risks to aquatic organisms, which are non-target organisms.

SOD is a cofactor that protects cells against oxidative stress; biochemical processes associated with detoxification are triggered in the liver, which contains tissues with oxidative reactions and maximum free radical formation (Wang et al., 2018; Aydın and Serdar, 2024). The increase in SOD activity during the early exposure periods (24–48 h) indicates an enhanced cellular response to elevated superoxide radicals generated under CPF stress. Similar increases in SOD activity have been reported in various aquatic species, suggesting that CPF induces the production of reactive oxygen species (ROS), triggering primary antioxidant defenses. The subsequent stabilization or decline of SOD activity across extended exposure periods likely reflects either cellular adaptation or enzyme inhibition at higher oxidative loads. Similar results have been demonstrated in other studies in the literature. Aydın et al. (2025), reported that the pesticide Gamma Cyhalothrin (GCH) caused changes in SOD activity in *Dreissena polymorpha*. Kumar et al. (2014), reported that CPF application caused an increase in CAT and SOD activities in *Chroococcus turgidus*. Tunca et al. (2023), stated that SOD activity increased with the effect of dimethoate and CPF in *Arthrospira platensis*. Mostafa, (2025) determined that SOD activity decreased in *Clarias gariepinus* individuals fed on *C. vulgaris* exposed to CPF. Yonar et al. (2022), determined that CPF caused an increase in SOD activity in *C. carpio*. Chen et al. (2016), stated that CPF increased the SOD activity of *Chlorella pyrenoidosa* and *Merismopedia* sp. Aydın et al. (2025), reported that dimethoate pesticide caused changes in SOD activities in *Pontastacus leptodactylus*.

Changes in catalase concentrations may result from the activation of the cell's antioxidant defense system. Increases or decreases in catalase concentration can be interpreted as a cellular response to neutralize the adverse effects of the pollutant (Wu et al., 2016). Decreases in CAT activity in *C. vulgaris* are also a cellular response. Mostafa, (2025) determined that CAT activity increased in *C. gariepinus* individuals fed *C. vulgaris* exposed to CPF. Yonar et al. (2022), determined that CPF caused an increase in CAT activity in *C. carpio*; Kumar et al. (2014), determined that CPF caused an increase in CAT activity in *C. turgidus*. Chen et al. (2016), reported that CPF increased CAT activity in *C. pyrenoidosa* and *Merismopedia* sp. Tawfeek et al. (2024), detected decreases in CAT activities in Nile tilapia fed *C. vulgaris* exposed to CPF.

GPx, a cytosol-based enzyme, is a tetrameric structure containing four selenium atoms. GPx provides the reduction of hydrogen peroxide and inorganic hydroperoxides. Yonar et al. (2022), determined that CPF caused decreases in GPx activity in *C. carpio*. Tawfeek et al. (2024), determined decreases in CAT activities in Nile tilapia fed *C. vulgaris* exposed to CPF. Zahran et al. (2020), examined the protective effect of *C. vulgaris* on *Oreochromis niloticus* individuals exposed to CPF and determined that GPx activity was inducible. Baruah et al. (2024), reported that CPF caused decreases in GPx activity in *Graesiella emersonii*. Samajdar et al. (2023), determined high GPx activity in *Labeo bata* due to CPF. Contrary to the literature, increases in GPx activity were observed in *C. vulgaris*, and these increases are thought to be dependent on the type of chemical applied, its concentration, and duration.

TBARS is a product of lipid peroxidation and plays an important role in determining oxidation and lipid damage under stress (Qian et al., 2012; Serdar et al., 2025). No significant change was observed in TBARS levels in *C. vulgaris* individuals after CPF exposure. However, increases and decreases in TBARS levels have been observed in the literature, and this is thought to be due to the model organism species and

application concentration. Tunca et al. (2023), reported that MDA levels increased under the effect of dimethoate and CPF in *Arthrospira platensis*. Mostafa, (2025) determined that MDA levels increased in *C. gariepinus* individuals fed CPF-exposed *C. vulgaris*. Yonar et al. (2022), determined that CPF caused statistically significant increases in MDA levels in *C. carpio*. Baruah et al. (2024), reported that CPF caused increases in TBARS levels in *G. emersonii*. Fernández et al. (2024), in their study where they applied microalgae fed CPF as a diet to mussels, determined increased MDA levels. Xing et al. (2012), reported that CPF and atrazine pesticides caused increases in MDA content in carp. Zhang et al. (2017), reported that CPF caused increases in MDA levels in carp, Nunes et al. (2018), in carp and zebrafish.

GSH, a key component of antioxidant defense, is a non-enzymatic scavenger. Glutathione protects cells from oxidative damage by reacting with free radicals and peroxides (Serdar et al., 2024). Mostafa, (2025) reported decreased GSH levels in *C. gariepinus* individuals fed *C. vulgaris* exposed to CPF. Xing et al. (2012), reported that CPF and atrazine pesticides caused decreases in GSH content in carp. Zhang et al. (2017), reported that CPF caused a decrease in GSH levels in carp. Jiao et al. (2019), observed a decrease in GSH levels in carp due to the effect of CPF. Samajdar et al. (2023), reported decreased GSH levels in *L. bata* and Mansour et al. (2022), reported decreased GSH levels in *Clarias gariepinus* due to the effect of CPF. Contrary to literature applications, the increases in GSH levels in *C. vulgaris* are thought to be due to the application concentration and duration.

Conclusions

In this study, experimental treatments on *Chlorella vulgaris* revealed that chlorpyrifos (CPF) exposure caused significant changes in antioxidant defense system enzyme activities (SOD, CAT, and GPx) and glutathione (GSH) levels, but no statistically significant changes in lipid peroxidation (TBARS). The findings indicate that CPF affects biochemical response mechanisms by triggering oxidative stress in *C. vulgaris* cells.

The observed changes in SOD, CAT, and GPx activities can be interpreted as a defense mechanism against the increase in intracellular reactive oxygen species (ROS). Conversely, the decrease in GSH concentration indicates that lipid peroxidation and oxidative damage are occurring at the cellular level. These results demonstrate that oxidative balance is disrupted when the antioxidant defense system is deficient in *C. vulgaris* cells exposed to CPF.

Therefore, it can be concluded that parameters such as SOD, CAT, GPx, TBARS, and GSH can be used as reliable biomarkers in assessing the toxic effects of CPF. Furthermore, the data obtained indicate that CPF and similar organophosphorus pesticides can have significant toxic effects not only on target pests but also on algae, the primary producers in aquatic ecosystems. This suggests that uncontrolled use of pesticides can disrupt the ecological balance of aquatic environments and lead to biocumulative effects throughout the food chain.

In conclusion, this study demonstrates that CPF exerts a toxic effect on *C. vulgaris* via oxidative stress, and that this effect can be monitored using biochemical markers. These findings support the use of microalgae as bioindicators in assessing pesticide-related pollution in aquatic ecosystems.

Ethical approval

The authors declare that this study complies with research and publication ethics.

Informed consent

“Not available”.

Conflicts of interest

There is no conflict of interests for publishing their study

Data availability statement

The authors declare that data are available from authors upon reasonable request.

Funding organizations

This study was supported by TUBITAK with project number 119Y592.

Author contribution

C. vulgaris propagation and maintenance were carried out by O.S., A.Ö., and T.D.

Experimental Design: Conducted by I.C.Ç.Ç, O.S., A.N.A, and T.P.A.

Biochemical Analyses: Conducted by O.S., A.N.A, and N.C.Y.

Statistical Analyses: Conducted by A.P.

Article Writing: Conducted by A.N.A.

Article Review: Conducted by O.S.

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