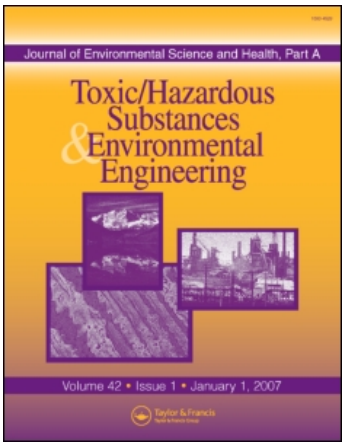


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# Effects of tetrabromobisphenol A on the green alga *Chlorella pyrenoidosa*

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Flow cytometry (FC) was used to determine effects of tetrabromobisphenol A (TBBPA) on the green alga *Chlorella pyrenoidosa* (*C. pyrenoidosa*) by evaluating esterase activity, membrane integrity, concentrations of intracellular reactive oxygen species (ROS) and chlorophyll *a*(Chl-*a*) auto-fluorescence. TBBPA can inhibit esterase activity. Esterase activity was inversely proportional with TBBPA with a 24 h $EC_{50}$ value of 3.13 mg TBBPA/L. After 48 h of exposure to TBBPA intracellular ROS was significantly greater than in the unexposed cells. TBBPA inhibited Chl-*a* fluorescence after 168 h. Concentrations of ROS were directly proportional to both magnitude and duration of exposure and was inversely proportional to cellular Chl-*a*. FC was useful as an integrated, ecologically relevant, measure of a functional response of the algae. The possible action pathway of TBBPA in *C. pyrenoidosa* is that TBBPA can cause toxic effects on esterase activity. As concentrations and exposure time increased, TBBPA change the ROS level in the internal. The role of anti-oxidative action is marked and significant at the duration of 48 h exposure, compared to the control. This suggested there was a redox cycle. TBBPA changes physiological status of cells, further decreased Chl-*a* fluorescence indicating inhibition.

**Keywords:** Toxicity, esterase activity, reactive oxygen species, chlorophyll *a*, productivity, flow cytometry.

## Introduction

Tetrabromobisphenol A (TBBPA) is produced in the greatest volume of the brominated flame retardants (BFRs) and used on more products than any other BFR.[1] TBBPA is also used as an intermediate in the production of TBBPA derivatives and brominated epoxy oligomers.[1] In addition, approximately 10% is used as an additive flame retardant in a wide variety of consumer products, including housings of domestic electric/electronic appliances.[1] TBBPA can be released into the environment through industrial effluents, landfill leachates, and found in aquatic sediments, soils, and biota samples.[2–8] TBBPA has a Lg Kow 4.5,[9] which indicates that it could be bioaccumulative. Concentrations as great as 63 ng TBBPA/g lipid weight (lw) have been measured in eel from freshwater and up to 245 ng TBBPA/g lipid in whiting (muscle) from the North Sea.[7]

While TBBPA has been reported to cause relatively little in vivo toxicity to mammals,[9] in vitro effects have been observed.[10] TBBPA have been found to be cause in vitro toxicity, including neuro-toxicity, immuno-toxicity and to be a thyroid hormone agonist and be weakly estrogenic.[11–20] In particular, the effects of TBBPA have recently been ascribed to interactions with cellular signaling pathways, especially with mitogen-activated protein kinases (MAPKs).[21] TBBPA causes relatively great acute toxicity to aquatic organisms such as mollusks, crustaceans, and fish,[21–23] but no information was available on the toxicity to photoautotrophic algae.

Photoautotrophic cells, consisting of only a cytoplasmic membrane and the cell wall, are exposed to pollutants and other discharges directly.[24] *C. pyrenoidosa*, a unicellular algal species common in eutrophic lakes, is a food source for many aquatic animals. It is extremely important for the functioning of aquatic systems that unicellular organisms

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like *C. pyrenoidosa* healthily grow as they are primary producers.

Conventionally, chlorophyll*a* (Chl-*a*) is measured for the evaluation of the physiological status of green algae.[25] In the present study, special dual-laser flow cytometry (FC) was employed to investigate the potential effects of TBBPA on esterase activity, membrane integrity, intracellular concentrations of reactive oxygen species (ROS) and Chl-*a*, all of which can provide information on potential sub-lethal effects and serve as early warning signals before morbidity or mortality is manifested.

## Materials and methods

### *Reagents and quantification*

TBBPA (purity>99%) was purchased from Aldrich Company. Concentrations of TBBPA in water were determined by use of high-performance liquid chromatography (HPLC). A Hewlett Packard (HP) 1100 HPLC with a HP DAD detector and an Agilent ZORBAX SB-C18 column (5$\mu$ m, 250 mm $\times$ 4.6 mm i.d) was used. Samples were filtrated through 0.45 $\mu$m filter paper. All other chemicals were commercially available products of analytical grade.

A stock solution of TBBPA was prepared in acetone and maintained in darkness at 0°C. Prior to each bioassay, stock solutions were brought to room temperature and used to prepare the final test concentrations and sterilized by filtration through a 0.22 $\mu$m paper filter paper. The acetone concentration in the medium including the solvent control was less than 0.1%. Concentrations of 2.67, 4.00, 6.00, 9.00, 13.50 mg TBBPA/L were confirmed to deviate less than 5% from the nominal concentrations that are reported here. Each treatment was prepared in quadruplicate along with control group and one solvent control group.

Flow cytometry (FC) conducted using previously published methods.[26–30] Flow cytometry measurements were made by use of a FACS Vantage SE flow cytometer (Becton Dickinson, USA) equipped with dual-laser bench and optics. Fluorescein-diacetate (FDA), Propidium iodide (PI), 2′,7′-dichlorodihydro-fluorescein-diacetate ($H_2$DCFDA) were purchased from Sigma Company. Caltag Counting Beads (Caltag Laboratories, Inc., CA) were used for algal cells density count.

### *Algal cultures*

*C. pyrenoidosa* was obtained from the Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences. The organisms were grown in batch culture in a BG-11 medium[31] under an illumination intensity of 1500 lux (12/12) at 26°C.[32] One hundred milliliters of the test solution was dispensed into 250 mL borosilicate glass Erlenmeyer flasks. All glassware were acid-washed in 10% concentrated $HNO_3$ before use. Sub-samples (5 mL) were immediately taken from each flask and filtered (0.22 $\mu$m). Exponentially growing cells were washed three times by centrifugation at 420 g 7 min and the concentrated cells added to the medium and incubated for 4, 24, 48, 72, 168, 192, or 216 h before FC analysis. At this substrate concentration, no toxicity due to the acetone solvent was observed.

### *Fluorescence staining*

The esterase activity, membrane integrity and ROS activity were determined by changes in FDA, PI and $H_2$DCFDA fluorescence, respectively. Each assay was performed on separate samples and fluorescence of $1\times 10^5$ cells was measured by flow cytometry. Intracellular esterase activity was determined by cleavage of FDA to produce fluorescein as a fluorescent probe[33,34] was used. Effects on membrane integrity were determined by use of PI.[33] Concentrations of both FDA and PI and durations of incubations were optimized previously.[33,35] Changes in FDA-dependent fluorescence as a result of TBBPA exposure were measured as the decrease of green fluorescence (FL1). A stock solution of 1000 $\mu$M FDA (dissolved in acetone) was added to a 3 mL solution of cells, to give a final concentration of 25 $\mu$M FDA. Cells were incubated with FDA for 8 min at a pH of 7.5 to 8.0. A stock solution of 1,000 $\mu$M PI was added to 3 mL aliquots of cells to give final concentrations of 10 $\mu$M PI.

The fluorescence of cells stained with PI was measured to study the cell viability.[25] Cells were incubated with PI for 15 min at a pH of 7.5 to 8.0. Effects of TBBPA on membrane integrity were determined by measuring the fluorescence emission of PI collected in the FL2 channel. Concentrations of intracellular ROS were determined by use of the 2′,7′-dichlorodihydro-fluorescein diacetate ($H_2$DCFDA) as described previously.[36,37] This lipophilic non-fluorescent indicator is easily de-acetylated to its fluorescent form (2′,7′-dichlorofluorescein; DCF).[38] Cells were harvested, washed once with phosphate buffer and incubated in the dark with 100 mM $H_2$DCFDA at 37°C for 60 min. Cells were then centrifuged and resuspended. Orange fluorescence emission of $H_2$DCFDA was collected in the FL1 channel.

### *Flow cytometry analysis*

Flow cytometry was conducted by use of a Coherent Innova 70-4 laser emitting at 488 nm; while fluorescence was collected by use of three-colored photomultiplier tubes with fluorescence emission filters (FL1 530/30 nm, FDA fluorescence or $H_2$DCFDA fluorescence; FL2 580/42 nm, PI fluorescence; and FL3 675/20 nm, Chl-*a* auto-fluorescence). Non-algal particles and dead cells were excluded from the analysis by use of a gating on FSC/FL3/FL4. The flow rate for FC analysis was 1.0 mL $s^{-1}$. Acquisition of data was made using the pulse height and log mode for all variables. The mean fluorescence

intensity (MFI) was obtained by converting the mean peak channel in FL1 into arbitrary fluorescence units and the results expressed as a percentage of the control. $EC_{50}$ of the esterase activity was obtained based on the MFI analysis. The results were expressed as a percentage of the control.[39] Calibration of the algal cell density was done by use of known quantities of Caltag counting beads.[35]

### *Statistical analysis*

The program Cell-Quest® from Becton–Dickinson was used to collect and analyze these signals. All the data were tested for homogeneity and normality of variance before analysis. After the data of esterase activity were pooled, TBBPA effects was expressed as an $EC_{50}$ value, i.e., the concentration of TBBPA required to reduce FDA fluorescence by 50% compared to the control.[33] The $EC_{50}$ value was calculated using linear interpolation methods of regression analysis with the statistical program CPIN. Data were expressed as mean ($n = 5$)± SD and analyzed using the SPSS for Win 12.5 computer program. ANOVA and Dunnett's t test were used to test differences between groups. The differences were regarded as statistically significant when $p < 0.05$.

## Results

### *Changes in esterase activity*

Cells were classified, based on green fluorescence as falling into one of three categories, representing three levels of general esterase activity. Cells in the M1 group exhibited less mean fluorescence intensity (MFI) green fluorescence while those in M2 represent control cells and those in M3 represent cells in which green fluorescence was greater than of the controls (Fig. 1). Exposure to 13.50 mg TBBPA/L for 24 h, resulted in the greatest lessening of green fluorescence relative to that of controls. The relative proportions of cells classified as M1, M2 and M3 varied as a function of duration (time) and intensity (concentration) of exposure (Fig. 1). Green (FA) fluorescence decreased after a 4 h TBBPA exposure.

As shown in Figure 2, decreases in FDA fluorescence were found before 168 h exposures as the TBBPA concentration increased. However, with longer exposure periods (192, 216 h), the effect of TBBPA on green fluorescence was not as consistent. The 24 h $EC_{50}$ value (based on mean activity states) was 3.13 mg/L (95% confidence limits 2.71–3.62). Green fluorescence did not increase as a function of time. Long-term exposure to TBBPA caused a decrease in green fluorescence.

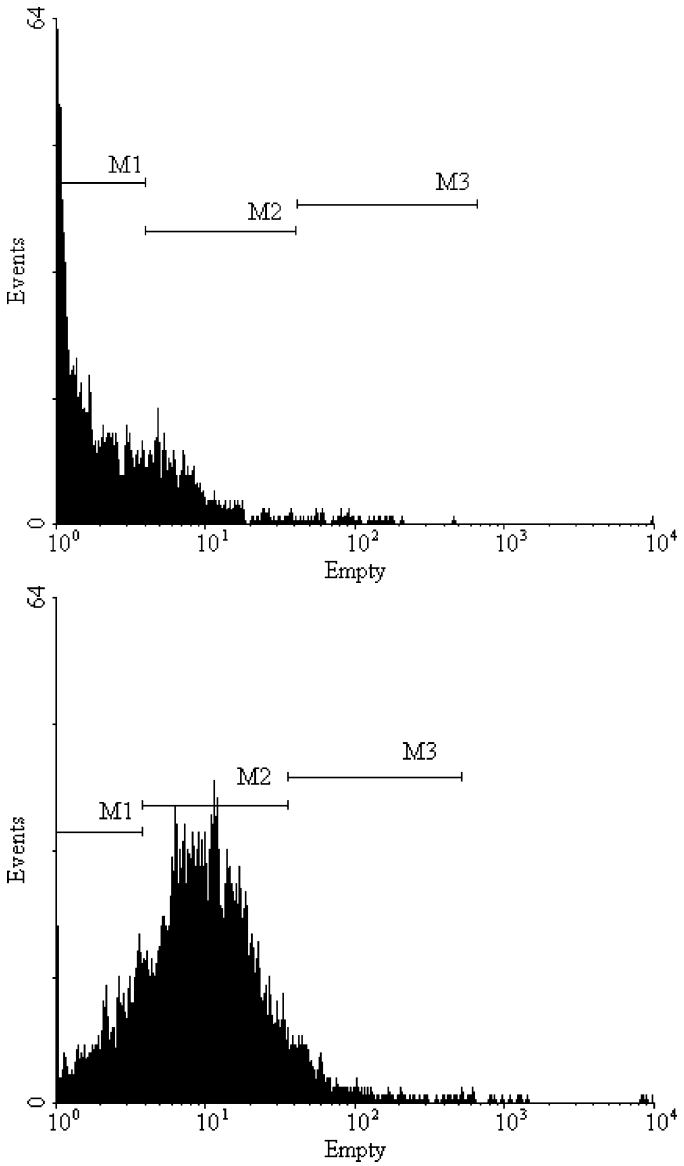


**Fig. 1.** FDA fluorescence histograms showing shifts in the profiles of *C. pyrenoidosa* control culture sample and a sample of a culture with TBBPA concentration of 13.50 mg/L after a 24-h exposure. M1 indicates a decrease in FDA fluorescence; M2 represents control cells; M3 indicates an increase in FDA fluorescence.

### *Changes in membrane integrity*

TBBPA caused statistically significant effects on membrane integrity of *C. pyrenoidosa*, as measured by changes in PI fluorescence relative to that of controls; however the effects were not rapid. After exposure to 13.50 mg TBBPA/L for 24 h the effect on membrane integrity was not significantly different from that of the controls and even after 48 h there was little effect on membranes caused by even the greatest concentration of 13.50 mg TBBPA/L (Fig. 3).

### *Changes in ROS level*

Concentrations of intracellular ROS responded differently as a function of both time and concentration of exposure

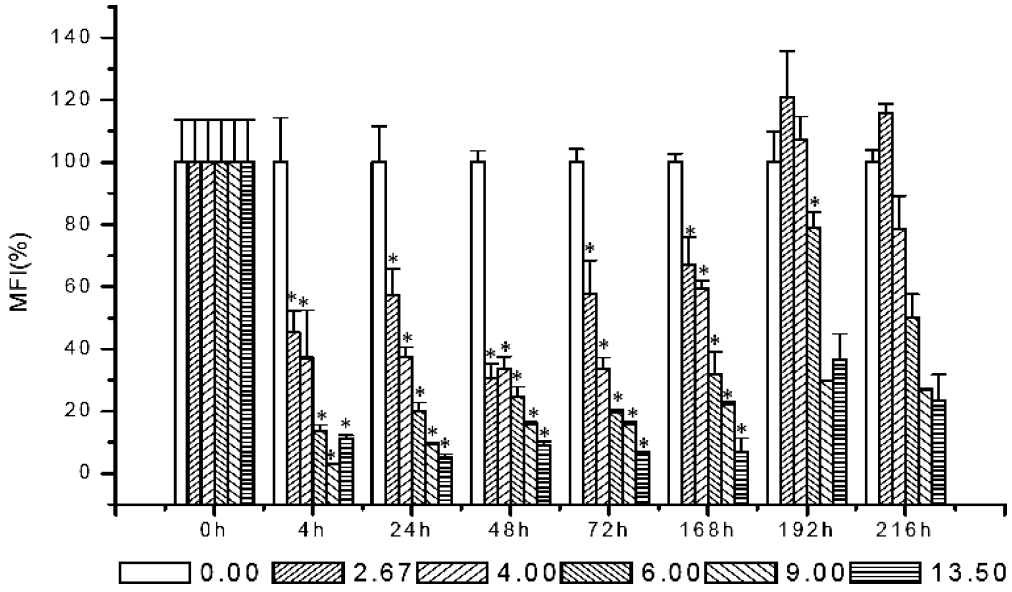


**Fig. 2.** Flow cytometry histogram showing shifts in mean fluorescence intensity (MFI) of *C. pyrenoidosa* after TBBPA exposure. Data points represent the mean± standard error of the mean (n = 5). *Significantly different from control, P< 0.05.

to TBBPA. Staining with $H_2$DCFDA showed a gradual increase in fluorescence as a function of time due to exposure to TBBPA exposure. The most rapid change in fluorescence occurred at the greatest concentration of 13.50 mg/L (Fig. 4). The effect of TBBPA on membrane integrity increased rapidly until 4 hr and then reached a maximum at 72 h with the effect decreasing thereafter (Fig. 5).

### *Changes of Chl-a auto-fluorescence*

Chl-*a* auto-fluorescence of *C. pyrenoidosa* was not significantly (P< 0.05) affected by exposure to TBBPA for 4 or 24 h (Fig. 6). In contrast, after exposure to TBBPA for168 h the intensity of Chl-*a* auto-fluorescence was significantly less than that of the unexposed cells (Fig. 6).

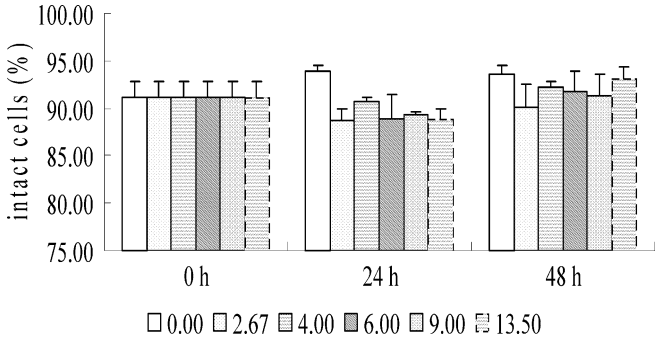


**Fig. 3.** Flow cytometry histogram showing shifts in PI fluorescence of *C. pyrenoidosa* after TBBPA exposure. Data points represent the mean± standard error of the mean (n= 5).

## Discussion

Even though there were no significant changes in the number of cells after short-term exposure to TBBPA, there were effects on esterase activity, membrane integrity, concentrations of intracellular ROS and Chl-*a* auto-fluorescence of *C. pyrenoidosa* changed continuously during TBBPA exposure. Cleavage of FDA, which can be readily absorbed by cells and metabolized by esterases, is a suitable probe to assess the viability of algal cells.[40,41] This technique is becoming a reliable endpoint in ecotoxicology.[42–46] The rate of FDA conversion to fluorescein is correlated

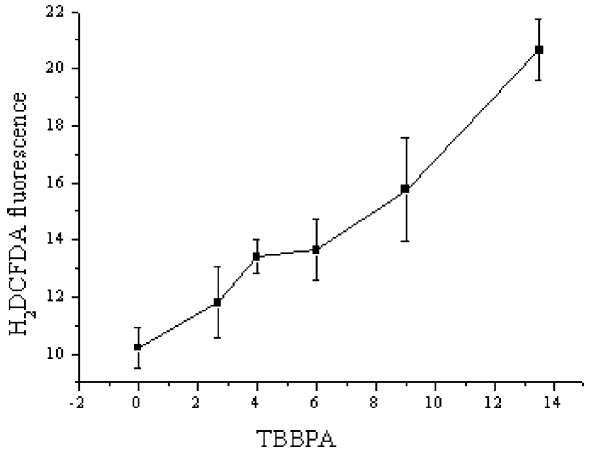


**Fig. 4.** Shifts in ROS production ($H_2$DCFDA fluorescence) of *C. pyrenoidosa* after different concentrations of 48 h TBBPA exposure. Data points represent the mean± standard error of the mean (n = 5).

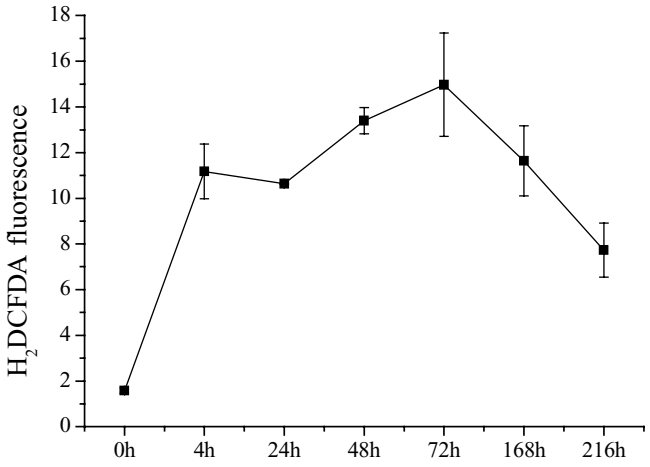


**Fig. 5.** Shifts in ROS production ($H_2$DCFDA fluorescence) of *C. pyrenoidosa* after different times of 4.00 mg/L TBBPA exposure. Data points represent the mean ± standard error of the mean (n= 5).

with photosynthesis[40] and nutrient limited growth,[41] which validates the use of this assay to assess the metabolic activity of phytoplankton cells. The effect can be classified as energy independent as enzyme will remain functional in cells as long as it is retained by the intact membrane and protected from the environment. TBBPA impaired esterase activity and caused toxic effects on it. Esterase activity has been suggested as a sub-lethal indicator of the physiological status of other alga.[33]

PI is a fluorescent dye that intercalates with double-stranded nucleic acids to produce red fluorescence when excited by blue light. It is unable to pass through intact cell membranes; however, when the cell dies the integrity of the cell membrane fails, and PI is able to enter and stain the nucleic acids.[47] PI has also been used to separate effects on esterase activity from cell membrane effects.[33] A fully intact cell membrane is impermeable to PI, so DNA will only be stained in cells that are dead or that have compromised membranes.[48,49] It will provide special information about the relationship between the cell cycle stage and susceptibility to TBBPA toxicity in FDA staining assay. The lack of uptake of PI suggests that the lesser green fluorescence was due to inhibition of intracellular esterase and not due to changes in membrane permeability and FDA uptake within 48 h exposure.

TBBPA-induced overproduction of ROS was also determined in toxicity test. TBBPA can induce oxidative stress by increasing the activity of glutathione reductase (GR), which has been reported in rainbow trout.[17] TBBPA could induce OH generation and result in oxidative damage in liver of *C. auratus*.[50] TBBPA enhanced ROS production in Human Neutrophil Granulocytes in a concentration-depended manner (1–12 mM), measured as 2′,7′-dichlorofluorescein diacetate amplified (DCF) fluorescence.[51] The results on ROS production by TBBPA was confirmed by lucigenin-amplified chemoluminescence. The TBBPA, induced formation of ROS was due to activation of respiratory burst, as shown by the NADPH oxidase inhibitor DPI (10 mM)[51] Oxidative stress directly damages proteins, amino acids, nucleic acids, porphyrins and phenolic substances, etc. In the present study, the TBBPA-induced ROS level of *C. pyrenoidosa* increased in a time-dependent manner, especially after 48 h exposure.

TBBPA change physiological status of cells further, decreased fluorescein fluorescence indicated impaired esterase activity or loss of cell membrane integrity. It might be related to the permeability of the cell membrane. When evaluating esterase activity, it was very important to assay membrane integrity. Berglund and Eversman[52] found that the amount of fluorescein that can accumulate in cells is dependent on the amount that leaks out of the cells due to the permeability of the cell membranes. But the majority of previous studies did not notice the effect. In this study,

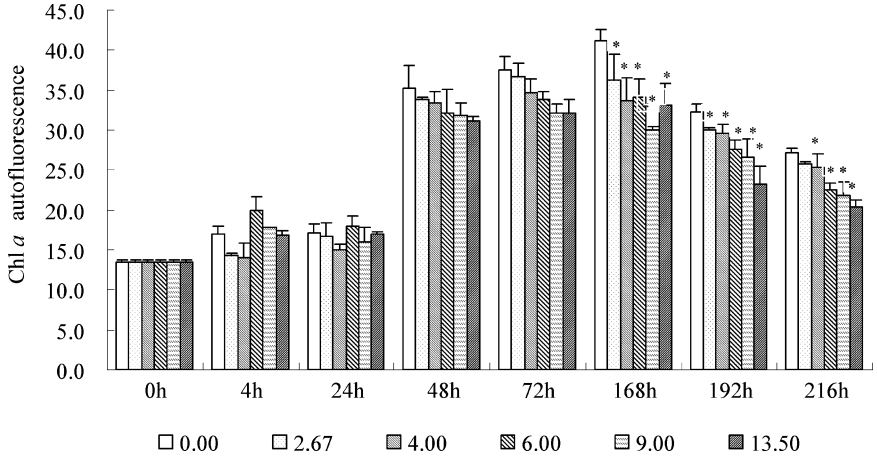


**Fig. 6.** Curves showing changes in Chl-*a* auto-fluorescence of *C. pyrenoidosa* cells after TBBPA exposure. Data points represent the mean± standard error of the mean (n= 5).

membrane integrity assay suggested that the change of the decrease in FDA fluorescence was mainly due to the inhibition of esterase activity.

Chl-*a* is an indicator of *C. pyrenoidosa* physiological status.[25,32,53] Measurement of the fluorescence of Chl-*a* provides information on the absorption, distribution and utilization of energy in photosynthesis.[39,54]

Chl-*a* auto-fluorescence of low esterase activity of *C. pyrenoidosa* did not show significant difference after a 72 h exposure: the nonviable cells still maintained their Chl-*a* auto-fluorescence. TBBPA can also induce *C. pyrenoidosa* cell dead, which have no metabolic ability. These results suggested that the assessments using Chl-*a* auto-fluorescence only as endpoint may underestimate the toxicity effect. In esterase activity bioassay, there are almost no studies seeking to investigate the differential sensitivity to compounds exposed to semi-lethal concentrations in pure algae population. The results elucidated the molecular basis for the adverse health effects associated with TBBPA exposure.

Concentration of pigments in algae is an indicator of their physiological status. The decrease in Chl-*a* fluorescence after 168 h exposure to TBBPA might be due to variations in the ratio of the pigment Chl-*a* resulting from adaptation to varying environmental conditions.[55]

The application of FC to ecotoxicology is enabling the development of more environmentally relevant toxicity tests with unicellular algae. This study extends those of previous studies on the effects of organic compound on phytoplankton, demonstrated the analytical potency of FC in allowing the integration effects of population, physiological, and biochemical levels to approach environmental biology.

The Chl-*a* auto-fluorescence assay and FDA/PI/$H_2$DCFDA staining procedures are useful approaches in the fast evaluation of the toxic effects on populations of unicellular phytoplankton, and may provide a rapid and special way of monitoring contaminant impacts in microalgae. In addition, FC has the ability to gather information simultaneously on the morphological, biochemical, and physiological effects of a toxicant and therefore may provide better insight into the mechanisms of toxicity.[33] Together with the present results, more efficient risk assessment procedures of TBBPA will be designed, integrating more flexible testing methods into the testing schemes that employ the bioavailability of TBBPA for more accurate estimate the effects. In the future, more tests including the chronic toxicity test, the long-term toxicity test in low dose exposure, and how the configuration of organisms changed will be used to assess the toxicology of TBBPA more accurately.

## Conclusions

According to these results, we proposed the possible action pathway of TBBPA in *C. pyrenoidosa*. Decreased fluoresence in FDA indicates impaired esterase activity. TBBPA can cause toxic effects on esterase activity. As concentrations and exposure time increased, TBBPA change the ROS level in the internal and impaired cell membrane integrity. The role of antioxidative action is marked and significant at the duration of 48 h exposure, compared to the control. This suggested there was a redox cycle. TBBPA changes physiological status of cells further, decreased PI fluorescence indicating loss of cell membrane integrity then decreased Chl-*a* auto-fluorescence indicating inhibition. These acute, sublethal endpoints may provide early warning signals before morbidity or mortality is visible.

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