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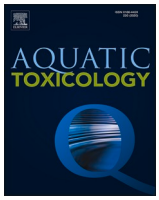

Toxicological effects, bioaccumulation, and metabolic pathways of tricresyl phosphate in *Scenedesmus obliquus*

Tianlie Luo [a,b,c], Jingjing Shi [a,b,c], Ping Zhang [a,b,c], Shuang Yang [a,b,c], Guo Liu [a,b,c,*], Willie J.G.M. Peijnenburg [d,e]

[a] State Key Laboratory of Geohazard Prevention and Geoenvironment Protection, Chengdu University of Technology, Chengdu 610059, China
[b] State Environmental Protection Key Laboratory of Synergetic Control and Joint Remediation for Soil & Water Pollution, Chengdu University of Technology, Chengdu 610059, China
[c] College of Ecology and Environment, Chengdu University of Technology, Chengdu 610059, China
[d] Institute of Environmental Sciences (CML), Leiden University, Leiden, 2300, RA, The Netherlands
[e] National Institute of Public Health and the Environment, Centre for the Safety of Substances and Products, Bilthoven, 3720, BA, The Netherlands

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ABSTRACT

In this study, *Scenedesmus obliquus* (*S. obliquus*) has been employed as a model organism to investigate the bioaccumulation, metabolism, and toxicity mechanisms of tricresyl phosphate (TCP). The results indicated that *S. obliquus* enhanced TCP degradation in water by 97 % after 14 days. The bioaccumulation factor of tricresyl phosphate in *S. obliquus* were calculated to be 8. When exposed to a high concentration of TCP (160 μmol/L), the algal growth rate was initially negative at 24 h, but gradually recovered over time. By 96 h, the inhibition rate was 64.74 % and the EC_{50} values was determined to be 86.41 μmol/L. Prolonged exposure to TCP substantially inhibited photosynthesis in *S. obliquus*, as indicated by a significant reduction in chlorophyll content. The addition of humic acid (HA), a representative substance of dissolved organic matter, exacerbated TCP toxicity by increasing ROS production, indicating a synergistic effect between HA and TCP. Conversely, a mixed nitrogen source reduced TCP toxicity. Four TCP metabolites were identified, resulting from hydroxylation, ketonization, hydrolysis, and ester bond cleavage. ECOSAR analysis revealed that these metabolites exhibit lower toxicity compared to TCP. These findings indicate that metabolic transformations within the algae may mitigate TCP toxicity, whereas HA significantly exacerbates TCP-induced oxidative stress. This study offers novel insights into the ecological risks of TCP in aquatic environments, especially in the presence of natural organic matter.

1. Introduction

Tricresyl phosphate (TCP) is a representative organophosphorus flame retardant commonly incorporated into paints, rubber, plastics, textiles, and upholstery (Wei et al., 2015). TCP is widely employed in Denmark, Norway, and other nations as a prevalent flame retardant across a diverse range of industries, with an annual consumption of up to 8000 tons (van der Veen and de Boer, 2012). Therefore, it has been frequently detected in water, soil, sediment, and air (Choo et al., 2018; Mo et al., 2019; Nantaba et al., 2021; Zhang et al., 2021a).

TCP has been frequently detected in the aquatic environment. For instance, TCP was detected at levels of 55 ng/L in samples collected from 16 municipal wastewater treatment plants in Austria (Martínez-Carballo et al., 2007). Furthermore, traces of TCP were detected even as far away as the Tiber River in Italy (Bacaloni et al., 2007). Unexpectedly, the maximum residual concentration of TCP in surface water reached 8100 ng/L in Lake Victoria, Uganda (Nantaba et al., 2021). This substantially heightens the risk of aquatic organisms experiencing negative impacts from pollutants. Alzualde et al. (2018) evaluated the developmental toxicity, neurotoxicity, cardiotoxicity, and hepatotoxicity of eight organophosphorus flame retardants (OPFRs) using zebrafish embryos, and they found that TCP produced adverse effects on several target organs. Therefore, we hypothesized that TCP may have toxic effects on algae, and there is still little information on this topic.

Algae play a vital role in aquatic ecosystems. They are primary trophic organisms that produce oxygen used by other organisms and capture carbon dioxide during photosynthesis. It is worth noting that algae can serve as a food and energy source for other organisms (Bai and

* Corresponding author.
E-mail address: liuguo@cdut.edu.cn (G. Liu).

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Acharya, 2017; Diaz et al., 2023). Oxidative stress is a major toxicity mechanism that severely affects the efficiency of scavenging reactive oxygen species (ROS) (Yin et al., 2020; Liu et al., 2023; Qi et al., 2024). When an organism is subjected to stress from harmful substances, ROS are produced in the body, and the organism enhances its superoxide dismutase (SOD) and catalase (CAT) synthesis to regulate antioxidant levels (Wu et al., 2016; Medvedeva et al., 2017). Overall, algae are commonly used as model organisms for assessing the toxic effects of chemicals, which can include an increase in algal ROS, a decrease in growth rate, and a decrease in chlorophyll-a levels (Che et al., 2021; Zhang et al., 2021b; Ding et al., 2023a; Kim et al., 2023; Wang et al., 2023). A previous study demonstrated that exposure to triphenyl phosphate (TPhP) leads to elevated levels of reactive oxygen species (ROS) in *Chaetoceros meülleri* after 96 h, ultimately resulting in cellular membrane damage (Wang et al., 2021). Similarly, Liu et al. (2019) found that tributyl phosphate (TBP), which is structurally similar to TCP, excessive ROS levels at exposed concentrations of 1.2 mg/L, causing oxidative damage in *Phaeodactylum tricornutum*, and accelerating apoptosis. To date, little is known about the toxic effects of TCP on algae. Examining the toxicity of TCP to algae is critical to understand its potential harm to freshwater ecosystems.

Revealing the toxic mechanisms of TCP can aid in the risk evaluation of these pollutants. Notably, the effects of organic pollutants when exposed to the aquatic environment vary, and the effects of co-existing substances in the environment must be considered when studying their toxic effects (Ding et al., 2019; Suzuki and shoji, 2020; Nanayama et al., 2021; Wang et al., 2022). Considering the physical environment, humic acid (HA) as a major component of natural organic matter (NOM) may influence the ecotoxicity of TCP. One study found that *Scenedesmus capricornus* was significantly inhibited when exposed to >2.0 mg/L humic acid (HA), while concentrations below this threshold promoted algal growth (Zheng et al., 2022). This is similar to those of Zhao et al. (2019a), who showed that HA could attenuate graphene-family-material (GFM)-induced membrane damage by reducing oxidative stress. However, HA does not always mitigate toxicity. Liu et al. (2020a) found that increasing levels of HA significantly increased the inhibitory effect on *S. obliquus*. Nitrogen is essential for the growth of microalgae (Ma et al., 2024). It is a vital element for the growth of algae and is frequently utilized to promote the development of microalgae. Researches have demonstrated that when algae are exposed to organic compounds, nitrogen as a nutrient interacts with these compounds, leading to unexpected outcomes for the algae. Zhang et al. (2013a) reported that ammonium (NH_4^+) reduced the growth rate of diatoms and rhodophytes, while nitrate (NO_3^-) reduced the photosynthetic activity of Rhodophyta (Martin-Jézéquel et al., 2015). Thus, it is important to consider their effect on the toxicity of organic pollutants.

When exogenous chemicals are introduced into the aquatic environment, they can be taken up by aquatic organisms through processes such as ingestion and absorption. Subsequently, these substances undergo transformation within the organisms. As the exposed hosts excrete these substances or experience apoptosis, they are subsequently released back into the environment, posing risks to other organisms such as zooplankton and fish. Prior research has demonstrated that some metabolites are more toxic than the original compounds, posing a significant threat to aquatic ecosystems (Shao et al., 2020; Jebalbarezi et al., 2022). Specifically, Liu et al. (2020b) used the ECOSAR program to assess the toxicity of tetracycline hydrochloride (TCH) and its intermediates. The results indicated that intermediate P6 exhibited significantly higher toxicity than TCH, with a 960-fold increase in acute toxicity to green algae compared to TCH.

TCP has been shown to bioaccumulate in humans (Zhao et al., 2019b), fish (Wang et al., 2019), and other animals through the food web (van der Veen and de Boer, 2012). A recent study has shown that the bioaccumulation factor of TCP in marine mussels is as high as 59,800 L/kg, which can be categorized as a very highly enriched compound, and its cumulative, toxic effects in aquatic organisms cannot be ignored (Mata et al., 2022). TCP readily accumulates in aquatic organisms and may undergo metabolic transformation (Hu et al., 2023). Chen et al. (2022) found that TCP inhibited the fertilization ability of juvenile Japanese medaka (*Oryzias latipes*), and TCP metabolites were detected in the liver. Reports also indicated that TCP may affect the chemical bonding of metabolites by influencing metabolic processes in the zebrafish liver and intestine (Chang et al., 2020). Researches on the toxicity and environmental fate of TCP in relation to algae, especially concerning metabolite production and associated toxicity, warrants greater attention.

In this study, *S. obliquus* was chosen as a model organism to investigate the toxic effects of TCP and examine the cumulative metabolic processes in algal cells under TCP stress. Metabolic pathways were proposed based on the intermediate metabolites identified in algae exposed to TCP. The toxicity of these metabolites was predicted. Investigating the toxic effects of multiple substances simultaneously provides insights into realistic exposure scenarios that are environmentally relevant. The potential modulation of TCP toxicity toward *S. obliquus* through interactions with environmental factors such as humic acid (HA) or nitrogen sources was systematically examined. It was hypothesized that co-exposure to HA or nitrogen sources might mitigate the toxicity induced by TCP. This research aims to elucidate novel mechanisms underlying the ecotoxicological effects of TCP.

2. Materials and methods

2.1. Chemicals

Tricresyl phosphate (TCP, 99 % purity) was purchased from Aladdin Biochemical Technology Co Ltd (Shanghai, China). The commercial HA used in these experiments was purchased from Chengdu Kelon Chemical Reagent Factory (Chengdu, China). TCP (0.289 g) was added to a 10 mL volumetric flask, and the solution was fixed to 10 mL with a dimethyl sulfoxide solution to prepare a reserve solution of 80 mmol/L TCP. This solution was sealed and stored at 4 °C in the dark. To ensure experimental stability, all experiments used this reserve solution. All other chemicals used in this study were analytical grade.

2.2. Algal cultures

S. obliquus (FACHB-12) was provided by the Institute of Hydrobiology, Chinese Academy of Sciences (Hubei, China). The purchased algae were inoculated into a sterile BG-11 medium with a pH value of 7.1 ± 0.3. The detailed composition of the BG-11 medium is shown in Table S1. The cultures were incubated in a light incubator at 25 ± 1 °C for 21 days with a light intensity of 4000 lux and a 12 h/12 h light-dark cycle, and they were shaken three times a day to prevent the growth of green algae cells against the walls. At the beginning of the experiment, the absorbance of the algal cells was measured for the first 21 days and the growth curve of *S. obliquus* was plotted based on the absorbance (Fig. S1). It was found that the algal cells had an exponential growth period in the first 7 days (with absorbances ranging from 0.008 to 0.250 L/(g·cm), which could be used as a basis for the subsequent experiments. The algal numbers were counted by hemocytometer under a light microscope, and the detailed method is provided in Text S1.

2.3. Degradation and accumulation of TCP in S. obliquus

2.3.1. Culture and collection of samples

In the cumulative experiments, TCP stock solutions with initial concentrations of 1 and 20 μmol/L (96 h-EC_{20} and 96 h-EC_{10}) were added to the culture medium, and then algal cells in the exponential growth phase were taken and added to 500 mL of BG-11 medium for the experiments (the initial concentration of algal cells was 1×10^5 cells/mL). To investigate the degradation kinetics and bioaccumulation potential of TCP in algae systems, *S. obliquus* was cultured in TCP-free

medium under controlled conditions, with a parallel blank control group established to account for background variability and ensure methodological rigor. The absorbance of algal cells at 684 nm was measured at 0, 1, 2, 3, 4, 7, and 14 d of the incubation process. The method for determining the weight of algal cells was referred to Ding et al. (2020a) and Zhang et al. (2013b). The dry weight of the algae was calculated from the density of algal cells: 1×10^5 cells/mL ≈ 0.0184 mg/mL.

2.3.2. Instrument conditions

In this experiment, TCP was quantified and analyzed using GC–MS (7890B, Agilent, USA). Before the start of the experiment, the samples were pre-treated, Text S2 describes how to prepare the samples. TCPs were quantified and analyzed by gas chromatography-mass spectrometry (GC–MS) with an inlet temperature of 280 °C, a scanning range of 50 ~ 400 m/z, a flow rate of 1 mL/min, a non-split flow rate, an injection volume of 1 μL, and a solvent delay of 4 min. The GC oven was kept at 100 °C for 1 min, then heated to 250 °C (15 °C/min), and then heated to 280 °C (2 °C/min), with a holding time of 1 min. The GC oven was held at 100 °C for 1 min, heated to 250 °C (15 °C/min), then to 280 °C (2 °C/min) and held for 1 min.

2.4. Toxicity assay

2.4.1. Growth inhibition

Considering the concentration range of TCP in real aqueous environments, the exposure concentrations in the acute toxicity test were set at 0, 0.1, 1, 10, 20, 40, 80, and 160 μmol/L, and the exposure time was 96 h. According to the data, the maximum concentration of TCP in water is 8100 ng/L, which is about 0.5 μmol/L. In order to simulate the exposure of real water environment, we first set the concentration of TCP at 0.5 μmol/L. Additionally, medium and high concentration groups (1 μmol/L and 20 μmol/L) were established for comparative analysis, and the exposure time was 21d. Experiments were performed when the green algae were in the exponential growth phase. The algal solution (3 mL) was sampled at a fixed time point, and the absorbance was measured at 684 nm.

After determining the absorbance of algal cells, it was necessary to analyze the average growth rate and growth inhibition rate of the green algae. The average growth rate (μ_t) and inhibition rate (I_c) of algal cells were calculated using Eqs. (1) and (2), respectively (Suzuki and shoji, 2020):

$$\mu_t = \frac{\ln N_t - \ln N_0}{t} \quad (1)$$

$$I_c = \frac{\mu_0 - \mu_c}{\mu_0} \quad (2)$$

where N_t denotes the biomass at time t, N_0 is the initial biomass, μ_0 is the average growth rate of the control group and μ_c is the average growth rate of the experimental exposed group.

2.4.2. Variation in chlorophyll content

Chlorophyll content was determined using the method of Li et al. (2021). In brief, 3 mL of algal solution was collected on days 0, 3, 7, 10, 14, 18, and 21, respectively, and the absorbance of algal cells at 684 nm was measured. The chlorophyll content of *S. obliquus* was determined by centrifugation at 10,000 rpm for 10 min, after which the supernatant was discarded and the cellular solids re-suspended in 3 mL of 95 % ethanol. The cells were subsequently incubated in darkness at 4 °C for 24 h and centrifuged again for 10 min, and then the supernatant was collected. The absorbance of the supernatant at 665 nm and 649 nm was then measured using a spectrophotometer (Youke, Shanghai). The chlorophyll concentrations were calculated as follows (Hanachi et al., 2022):

$$\text{Chlorophyll} - \text{a}(\text{mg}/\text{L}) = 13.70 \times OD_{665} - 5.76 \times OD_{649}$$

$$\text{Chlorophyll} - \text{b}(\text{mg}/\text{L}) = 25.80 \times OD_{649} - 7.60 \times OD_{665}$$

$$\text{Chlorophyll}(\text{mg}/\text{L}) = 6.10 \times OD_{665} + 20.04 \times OD_{649}$$

2.4.3. Changes in antioxidant enzyme activity

The algal solution (10 mL) was collected at specific times (0, 24, 48, 96 h and 3, 7, 10, 14, 18, 21 d) and centrifuged at 1000 rpm for 10 min (4 °C). The supernatant was then discarded, algal cells were collected. We added 1 mL of PBS (0.01 mol/L, pH 7.4) to the algal cells, mixed it well, centrifuged as stated previously, discarded the supernatant, and washed the precipitate three times with PBS. After that, we added 0.5 mL of PBS to the precipitated algal cells, mixed well, and transferred the algal solution to a Lysing Matrix D tube. The algal solution was placed into a cryomiller for 20 min (2000 rpm, −20 °C) for cell fragmentation. Then, the algal liquid was removed from the lysing tube, loaded into 1 mL centrifuge tube, and centrifuged (4 °C) to obtain the supernatant. The total protein (TP) content, SOD activity, and CAT activity were quantified using corresponding assay kits (Jiancheng Bioengineering Institute, China).

2.5. Toxic effects of environmental factors

2.5.1. Influence of HA on toxic effects

To more accurately simulate real water environments, we established exposure concentrations of 1 mg/L and 10 mg/L (ambient concentration) of HA in the presence of TCP, and included a blank control group. The dimethyl sulfoxide (DMSO) content in each group was maintained below 0.1 % (v/v). Algal cells (30 mL) at an initial concentration of 1×10^5 cells/mL were placed into a sterilized conical flask (50 mL), and the absorbance at 684 nm was measured using a spectrophotometer at 96 h. We used an enzyme marker as well as a ROS reagent to measure the ROS content of the cells at 96 h. The fluorescence intensity of the samples was observed using fluorescence microscopy. Text S3 in the supplementary material contains a thorough description of the procedures used to determine the ROS content in *S. obliquus*.

2.5.2. Influence of nitrogen sources on toxic effects

To study the effects of different nitrogen sources on the cytotoxic effects of TCP on algal cells, TCP was tested at concentrations of 0, 1, and 20 μmol/L. The nitrogen was placed in BG-11 medium at a concentration of about 247 mg/L, and the experiments used NH_4^+-N, NO_3^--N, and NO_2^--N as the nitrogen sources at a 1:1:1 ratio. To control for the variables, additions were made as shown in Table S2 (the BG-11 nitrogen source is $NaNO_3$). During the exponential growth period, a certain amount of algal liquid was taken and added to various types of nitrogen source media so that the initial concentration of algal cells was 1×10^5 cells/mL.

2.6. Metabolites identification and prediction toxicity

Taking 10 mL of algal solution, was centrifuged three times, placed the cell precipitate into a refrigerator, and freeze-thawed the precipitate three times. Then, 4 mL of methanol/dichloromethane (2/1, v/v) was added, and the mixture was treated with an ultrasonic cleaner for 1 h. It was then centrifuged at 5000 rpm for 10 min (4 °C) on a high-speed refrigerated centrifuge. Finally, the supernatant was removed for measurement. The algae cell samples were characterized using LC-MS (Thermo Fisher Scientific Q Exactive, USA). The column used was Hypersil GOLD (100 mm × 20 mm × 3 μm, USA). See Text S4 for details on the instrumental analysis of LC-MS. The metabolite toxicity of TCP was predicted using the ECOSAR (US EPA) model, which is an ecological constitutive relationship model (Karci, 2014; Thomaidi et al., 2015).

2.7. Data analysis

All experiments were performed in triplicate. Significant differences in the growth rate of *S. obliquus*, Chlorophyll content, and enzyme activity between the control and TCP-treated groups were evaluated using one-way analysis of variance (ANOVA) in SPSS 16.0, and it was considered as significant difference at $p < 0.05$. All data satisfy normal distribution and homogeneity.

3. Result and discussion

3.1. Dissipation of TCP in culture media

In the initial experiment, the lower-exposure TCP concentration with algae accelerated the removal rates (Fig. 1a). Specifically, 1 μmol/L TCP was removed when the algae were present at 1 d, and the removal rate was up to 70 %. However, only approximately 30 % removal of 20 μmol/L TCP was detected in algae cultures. On day 14, removal rates of 97 % (with algae) and 92 % (without algae) were achieved in the low concentration TCP exposure group, and removal rates of 96 % (with algae) and 79 % (without algae) were achieved in the high concentration. It can be contrasted that algae play a role in TCP degradation, especially at high concentrations (20 μmol/L), and their degradation efficiency is significantly increased. Similar results have been found in previous studies (Chu et al., 2022). The observed phenomenon may be attributed to the significant concentration gradient between the interior and exterior of the algae cells. This gradient creates a driving force that facilitates the influx of more TCP from outside the algal cell into the alga, promoting its degradation and transformation. This phenomenon suggests that in real environments, the algae may be more in terms of ecological remediation or toxicity mitigation under long-term exposure.

Fig. 1b shows the first-order kinetic fitting curves for the degradation rates of TCP in different matrices, and the details of the fitting parameters are shown in Table S3. These data suggest that *S. obliquus* can have a positive effect on the degradation of TCP. Some studies have shown that aquatic plant algae can reduce the toxicity of chemicals and degrade aquatic pollutants. As an example, algae can mitigate antimicrobial toxicity by degrading sulfamethoxazole (SMX) into transformation products (TPs) with lower ecotoxicity (Zhang et al., 2023). In addition, the removal of TCP increased with its increasing exposure time. The findings presented here exhibit a resemblance to the outcomes reported by Lu et al. (2022) and Ding et al. (2023b).

3.2. Accumulation of TCP in S. obliquus

In the 1 μmol/L TCP treatment, the intracellular concentration of algae decreased with increasing time (Fig. 1c). The intracellular concentration of TCP in algae significantly decreased from 7.74 μmol/g at 1 d to 0.13 μmol/g at 14 d. In the 20 μmol/L TCP treatment, the content decreased in the first 3 d and then increased to approximately 14 μmol/g at 7 d. This result is consistent with the results reported by Ding et al. (2020b), showing changes in the galaxolide concentration in algae. In general, both TCP concentrations showed a decreasing trend in algal cells, suggesting that TCP of the algal cells was transformed. The ratio of intracellular to extracellular concentrations in algal cells was determined (Fig. 1d). As exposure time increased, the ratio decreased in the first 2 d; then, it increased to 8 with 1 μmol/L TCP. A similar result was obtained at a higher exposure concentration. The upward trend in the

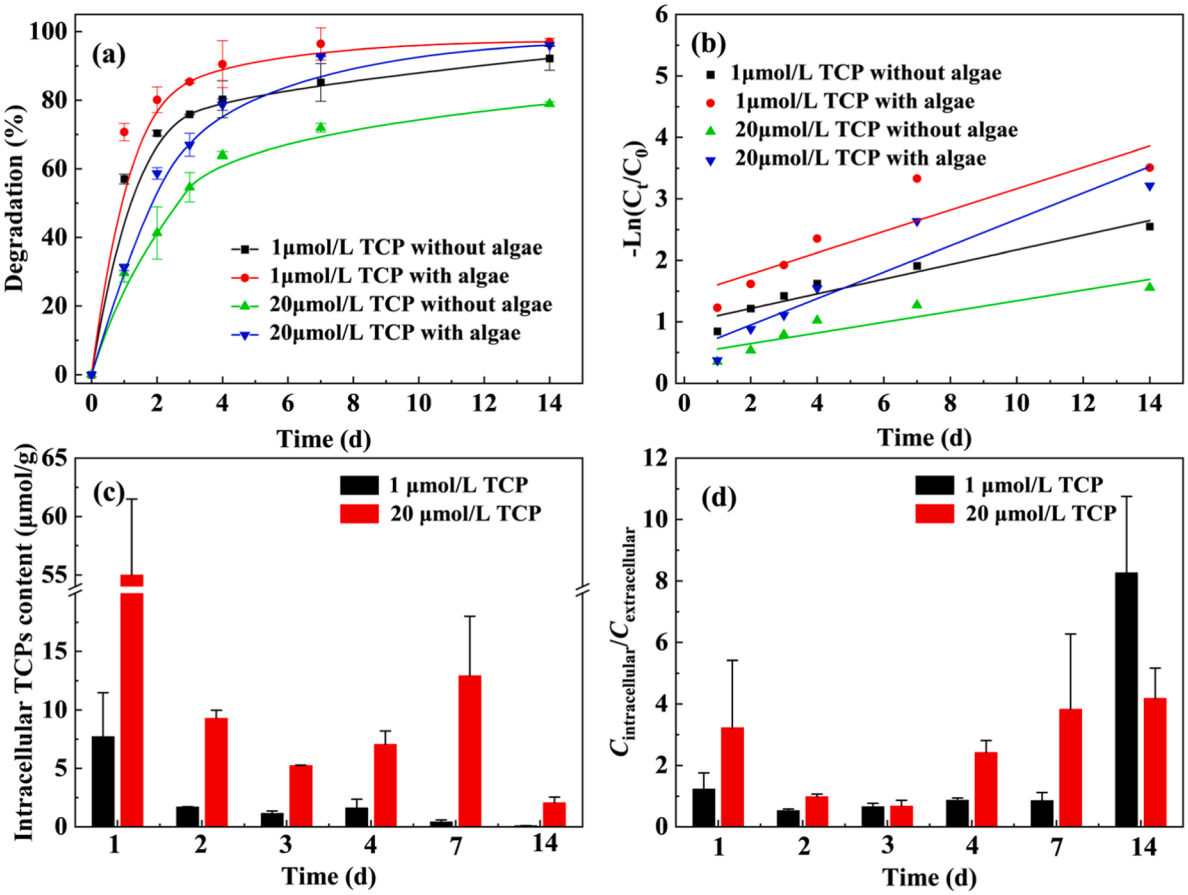

Fig. 1. (a) Removal rates of TCP in algae-containing and algae-free substrates. (b) First-order kinetic fitting. Error bars represent standard deviations ($n = 3$). (c) Variation in the concentration of TCP in algal cells, (b) Intracellular/extracellular concentration ratio. Over time in an exposure experiment. Error bars represent standard deviations ($n = 3$).

ratio of intracellular to extracellular concentrations indicated the accumulation of TCP in algal cells.

3.3. Effects of TCP on the growth of S. obliquus

The low concentration of TCP (0.1 μmol/L) had little effect on the growth of *S. obliquus* (Fig. 2a and b). However, the other concentrations of TCP significantly inhibited the cell density and mean growth rate of *S. obliquus*, and the inhibition increased as the concentration of TCP increased.

The experimental groups with TCP concentrations of 80 and 160 μmol/L had negative algal cell growth rates at 24 h. This shows that high concentrations of TCP were lethal to algal cells at the beginning of the experiment, while the average growth rate of algal cells tended to increase continuously with time. At 48 h, the average growth rate became positive; at 96 h, the average growth rate continued to increase, which indicated that the average growth rate of the algal cells had a tendency to increase with the increase of time; and at the same time, in the experimental group of 1~40 μmol/L, the average growth rate of the algal cells increased in comparison with the average growth rate of the algal cells at 48 h. It was speculated that the toxicity of TCPs would decrease with the increase of the exposure time.

The data were plotted to produce a concentration-response fitting curve (Fig. S2), which shows that the 24-h EC_{50} of TCP against *S. obliquus* was 29.77 μmol/L, the 48-h EC_{50} was 30.80 μmol/L and the 96-h EC_{50} was 86.41 μmol/L. The EC_{50} value increased with time, demonstrating that the toxicity of TCP to *S. obliquus* decreased with time. These data are consistent with the results of the cumulative experiments. In the chronic toxicity experiment, the average growth rate of *S. obliquus* had a decreasing trend with increasing TCP exposure concentration, similar to the results of the acute toxicity experiment (Fig. 2c and d).

3.4. Changes in CAT, SOD contents, and chlorophyll content

The SOD activity increased substantially with increasing TCP concentrations after 24, 48, and 96 h of exposure (Fig. 3a). Specifically, the SOD activity increased from 19 U/ugprot in the control to 100 U/ugprot with 20 μmol/L TCP after 24 h. The activity was concomitantly determined at 60 U/ugprot and 35 U/ugprot with 20 μmol/L TCP after 48 and 96 h, respectively. At 24, 48, and 96 h, the SOD activity was significantly increased in the 20 μmol/L experimental group compared with the control ($p < 0.001$). This demonstrates that after exposure, 20 μmol/L TCP acted on *S. obliquus* and still produced excess superoxide.

CAT is used to break down the hydrogen peroxide produced by superoxide dismutase and other enzymes into water and oxygen. A similar increase was also detected in the activity of CAT. With the increase in TCP concentration, the CAT activity of *S. obliquus* gradually increased (Fig. 3b). In particular, the CAT content in *S. obliquus* was 0.02, 0.03, 0.08 and 0.09 U/ugprot at concentrations of 0, 1, 10 and 20 μmol/L,

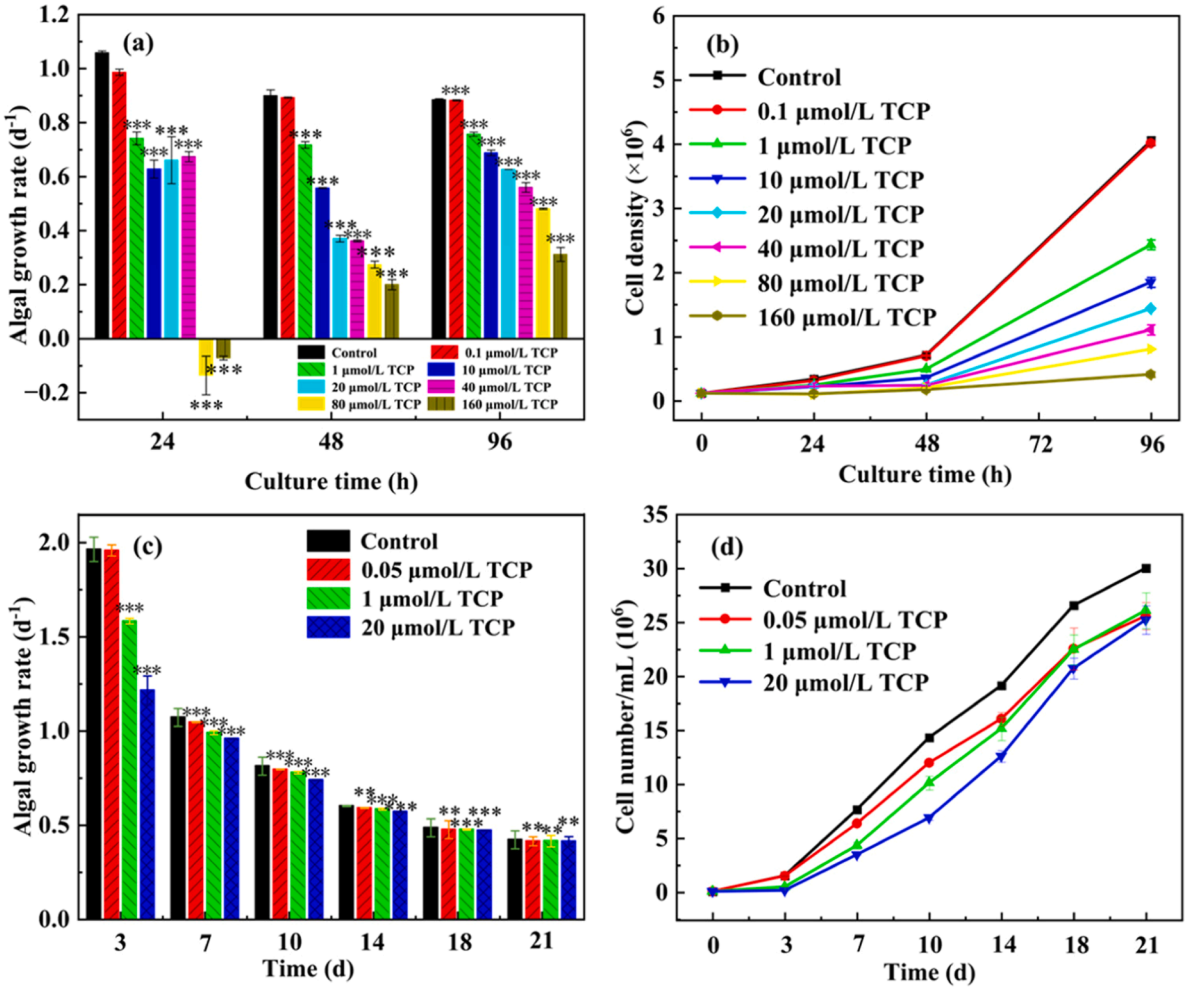

Fig. 2. (a) Mean growth rate of *S. obliquus*, (b) Effect of TCP on the growth of *S. obliquus* (the exposure period is 96 h), (c) Mean growth rate of *S. obliquus*, (d) Effect of TCP on the growth of *S. obliquus* (with an exposure period of 21 days). Error bars represent standard deviations ($n = 3$). *, ** and *** indicate statistical significance levels ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) compared with the control.

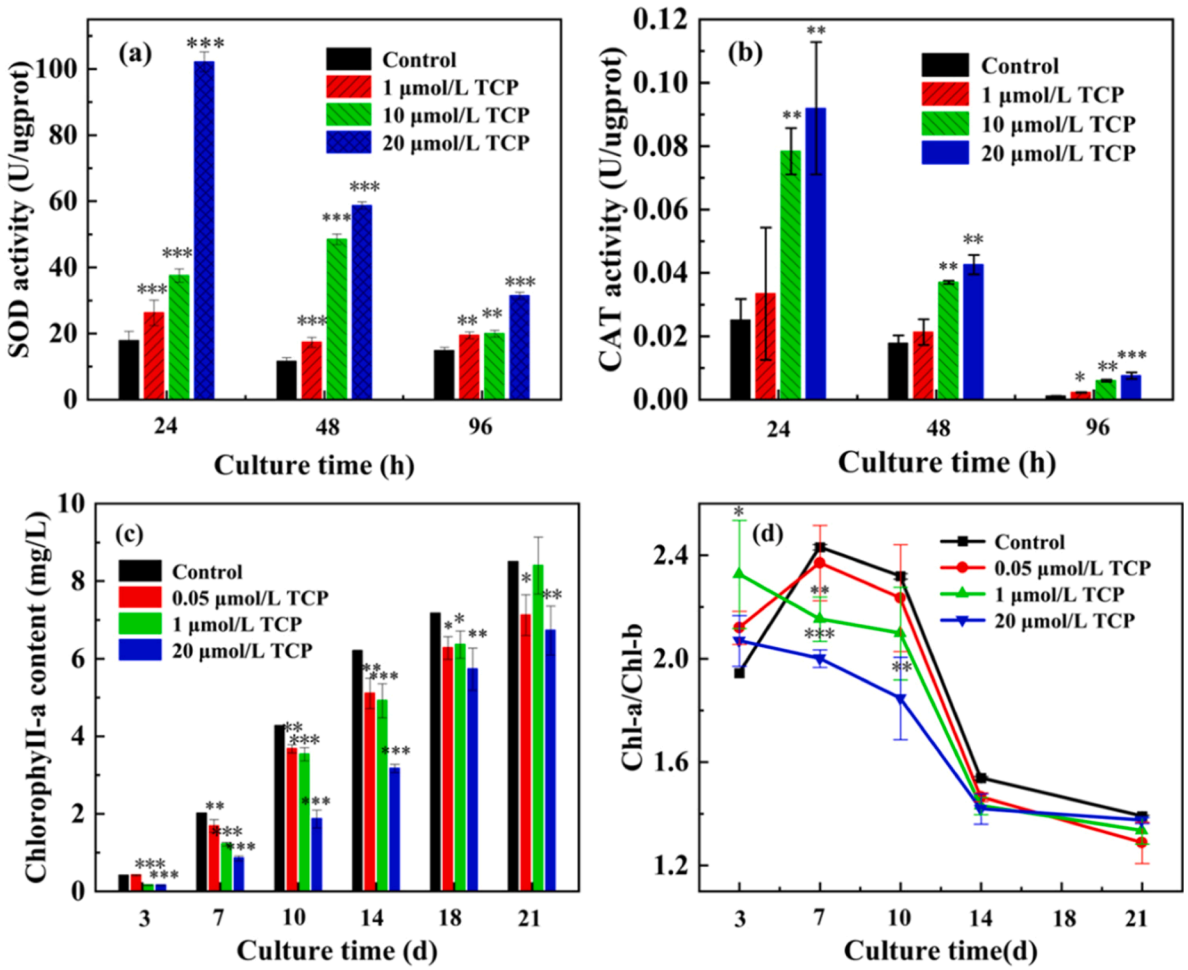

Fig. 3. Changes in SOD (a) and CAT (b) activities within *S. obliquus* at 24, 48, and 96 h, and changes in Chlorophyll-a content (c) and Chlorophyll-a/Chlorophyll-b ratio (d) in algal cells over 21 days. Error bars represent standard deviations ($n = 3$); *, ** and *** indicate statistical significance levels with the control ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

respectively, after 24 h of exposure. In general, the CAT and SOD activities showed an overall decreasing trend, which is consistent with the conclusion above, indicating that the toxicity of TCP to algal cells decreased with an increase in exposure time.

The chlorophyll-a content in algal cells was measured for 21 d of the chronic experiment (Fig. 3c). In the initial experimental, the cellular chlorophyll-a content gradually decreased with increasing TCP concentration, and the decrease in chlorophyll-a may be attributed to TCP-induced disruption of the photosynthesis system or accumulation of ROS (Zhang et al., 2021c). Changes in chlorophyll content may be a dynamic response of algae to oxidative stress. TCP exposure may lead to accumulation of ROS, which destroy chlorophyll molecules while inducing algae to adjust the balance between chlorophyll synthesis and degradation. The decrease in photosynthetic pigments may also be caused by the peroxidative decomposition of pigments and membrane lipids by ROS (Huang et al., 2012). The changes in the chlorophyll-a/chlorophyll-b ratio in algal cells are shown in Fig. 3d. On day 3, the ratio was higher in the experimental groups of 0.05 μmol/L, 1 μmol/L and 20 μmol/L TCP than in the control group. This was probably due to the protective mechanism of algal cells against photooxidative damage at the beginning of the experiment (Pancha et al., 2015).

3.5. Toxic effects of TCP on algae in the presence of HA and nitrogen sources

The cell densities of *S. obliquus* exposed to HA and TCP at 96 h are shown in Fig. 4a. When the concentrations of TCP were 0 and 0.1 μmol/L, the changes in algal cell densities were not significant with the increase in HA concentration. When the concentrations of TCP were 1 and 10 μmol/L, the densities of algal cells gradually decreased with the increase in HA concentration and significantly decreased compared with those of the group not exposed to HA ($p < 0.05$, $p < 0.01$, and $p < 0.001$). When the concentrations of TCP were 20, 40, 80, and 160 μmol/L, 1 mg/L HA had little effect on the growth of algal cells exposed to TCP, and 10 mg/L HA significantly decreased the cell density of *S. obliquus* compared with the group not exposed to HA. Obviously, when the concentration of TCP was 160 μmol/L, the 10 mg/L HA-exposed group had a 15.96 % increase in the growth inhibitory toxicity of TCP on algal cells compared with the control group (Fig. 4b).

At the same time, the total amount of ROS in the cells of *S. obliquus* induced by 10 mg/L HA was significantly higher than that of the control group (Fig. 4c), suggesting that HA promoted the production of total ROS. Fluorescence bioimaging provided direct evidence of the interaction between TCP and algae (Fig. S3). These results are similar to those reported previously. Geng et al. (2024) found that the presence of HA exacerbated the oxidative stress of perfluorooctane sulfonate (PFOS) in mussels with a significant increase in malondialdehyde (MDA). Similarly, HA promotes *Selenastrum capricornutum* growth at a relatively low concentration of 2.0 mg/L but inhibits algal growth at high concentrations. This may be the result of excessive ROS production, which causes toxic effect (Zheng et al., 2022).

Under the influence of different nitrogen sources, the SOD activity of

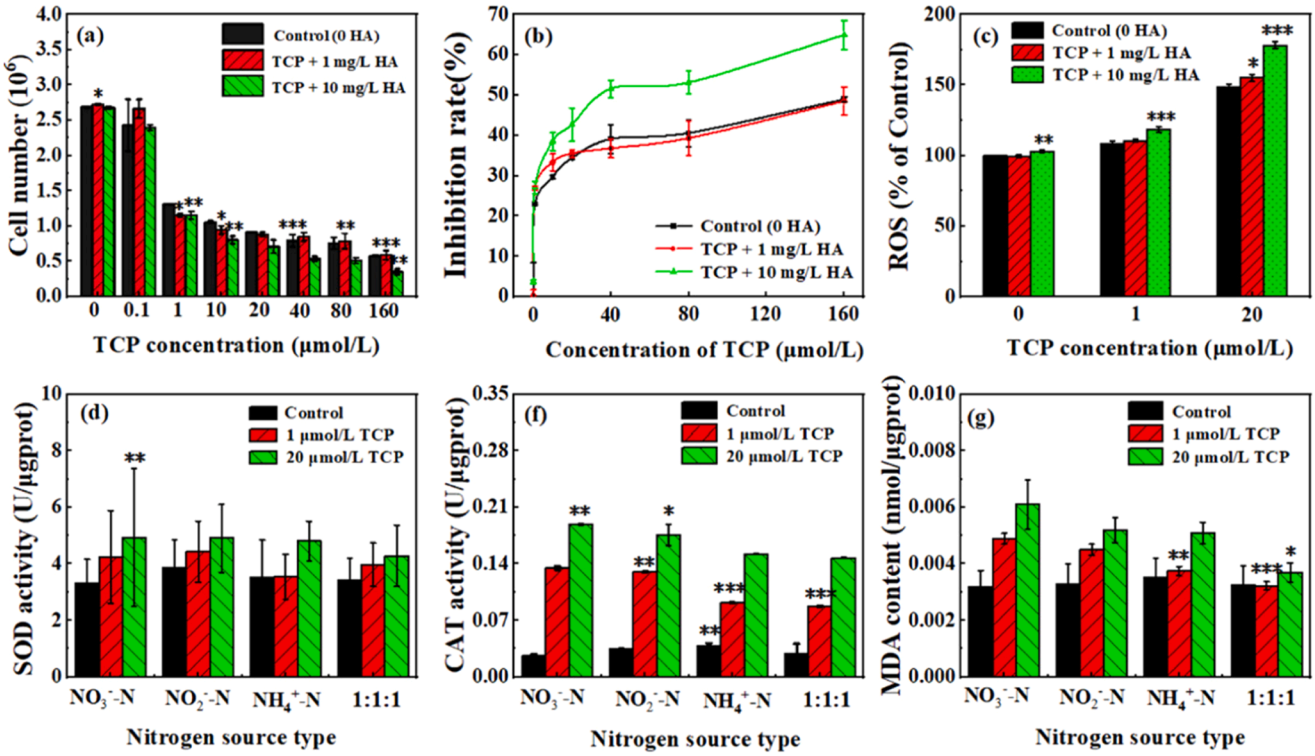

Fig. 4. Cell density (a), Growth inhibition (b), and ROS (c) of *S. obliquus* at 96 h upon exposure to HA and TCP. Changes in SOD (d), CAT (f), and MDA (g) activities of algal cells exposed to 1, 20 μmol/L of TCP for 96 h under the influence of different nitrogen sources. Error bars represent standard deviation ($n = 3$); *, ** and *** indicate the level of statistical significance concerning the control group ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

algal cells showed an increasing trend with the increase of TCP concentration after 96 h of exposure (Fig. 4d). The CAT activity of algal cells at 96 h showed an increasing trend with the increase of TCP concentration (Fig. 4f), in which the NO_2^--N and NH_4^+-N nitrogen source groups were significantly higher than the NO_3^--N nitrogen source group in the group without TCP. Furthermore, CAT activity in the NO_3^--N nitrogen source group was overall higher than that in the NO_2^--N nitrogen source group, the NH_4^+-N nitrogen source group, and the mixed nitrogen source group.

The changes in MDA content in algal cells at 96 h are shown in Fig. 4g. MDA had an increasing trend with the increase in TCP concentration. The MDA content was slightly higher in the control group without TCP, the NO_2^--N nitrogen source group, NH_4^+-N nitrogen source group, and mixed nitrogen source group than in the NO_3^--N nitrogen source group, but the differences were not significant. In the experimental group of 1 μmol/L TCP, the NH_4^+-N nitrogen source group was

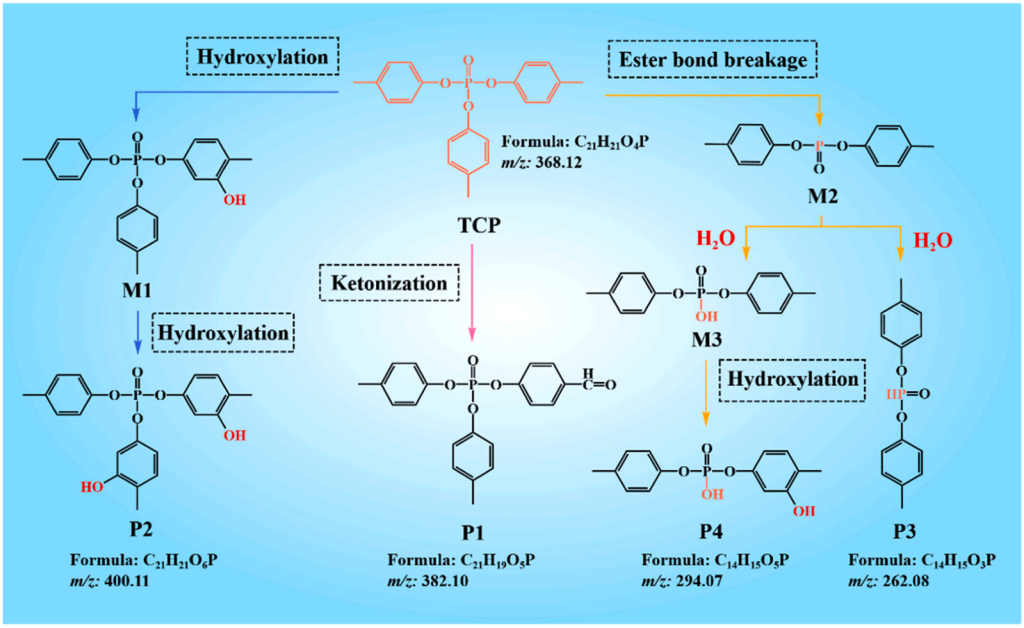

Fig. 5. Possible metabolic transformation pathways of TCP in *S. obliquus*.

significantly lower than the NO_3^--N nitrogen source group ($p < 0.01$), and the mixed nitrogen source group was significantly lower than the NO_3^--N nitrogen source group ($p < 0.001$). These results verify that NH_4^+-N and mixed nitrogen sources had a mitigating effect on the cytotoxicity of the TCP to algae.

3.6. Hypothesized metabolic pathways of TCP in the presence of S. obliquus

This study demonstrated that TCP released into the aqueous environment can accumulate in algal cells. To understand the fate of TCP in the organism, the metabolites of TCP in *S. obliquus* were identified using LC-MS high-resolution mass spectrometry. Four metabolites—P1, P2, P3, and P4—were produced by *S. obliquus* during the degradation of TCP (Fig. S4). Based on the detected metabolites of TCP, a metabolic transformation pathway of TCP in *S. obliquus* was postulated (Fig. 5).

The metabolism of TCP in algae is divided into three main aspects: ketonization, hydroxylation, and ester bond breaking. The aryl phosphate branched chain terminal carbon and the neighboring and interstitial positions of the benzene ring have high reactivity (Wang et al., 2017). Therefore, TCP undergoes a ketonization reaction catalyzed by biological enzymes of algae to generate compound P1. The benzene ring of TCP will be hydroxylated to form intermediates under the action of bio-oxygenase, and the intermediates continue to hydroxylate to generate the metabolite P2 (dihydroxy TCP). Van den Eede et al. (2015) reported similar findings in a study on the metabolism of OPFRs in zebrafish. Tris(2-butoxyethyl) phosphate (TBOEP) can be oxidized to monohydroxy Bis(2-butoxyethyl) phosphate (BBOEP) by cytochrome P450 enzymes in zebrafish. The C—O bond in TCP is easily damaged by enzyme attack and undergoes ester-bond breakage, which is transformed into the intermediate product M2. M2 in the presence of water molecules produces the metabolic product P3 and the intermediate product M3 (dimethylphenyl phosphate). TCP is degraded to dimethylphenyl phosphate in water and soil, but little M3 was detected. The hydroxylated metabolite P4 of dimethylphenyl phosphate was also detected, and the combined analysis suggested that most of the M3 produced might have been hydroxylated to P4. The toxicity of metabolites P1, P2, P3, and P4 has not been previously reported. In addition, environmental risk assessment of metabolized intermediates is lacking. In this study, the ECOSAR prediction model was used to estimate the aquatic toxicity of TCP metabolites to *S. obliquus*.

3.7. Metabolites toxicity assessment

The toxicity of intermediates in the degradation of TCP was evaluated by calculations using the ECOSAR program in EPISuite (Sanderson et al., 2003; Graumans et al., 2024; Kumari and Kumar, 2022). The results showed that the metabolite P1 was slightly more toxic to daphnia than the ontogenetic contaminant. In the chronic toxicity data for P1, the chronic toxicity concentration to fish (6.4×10^{-3} mg/L) was also slightly greater than that of the ontogenetic contaminant (4.3×10^{-3} mg/L) (Fig. 6, Table S4). However, that for *daphnia* was less than that for the ontogenetic contaminant (0.037 mg/L), and the LC_{50} for P1 was less than that for the ontogenetic contaminant.

Furthermore, metabolites P2, P3, and P4 are significantly less toxic than TCP. The chronic toxicity data of metabolite P3 showed that its chronic toxicity concentration to green algae (1.160 mg/L) was 28 times higher than that of TCP (0.042 mg/L), which proved that the toxicity of P3 was much smaller than that of TCP; the metabolite P4 had a similar pattern, and its chronic toxicity concentration to *daphnia* (0.171 mg/L)

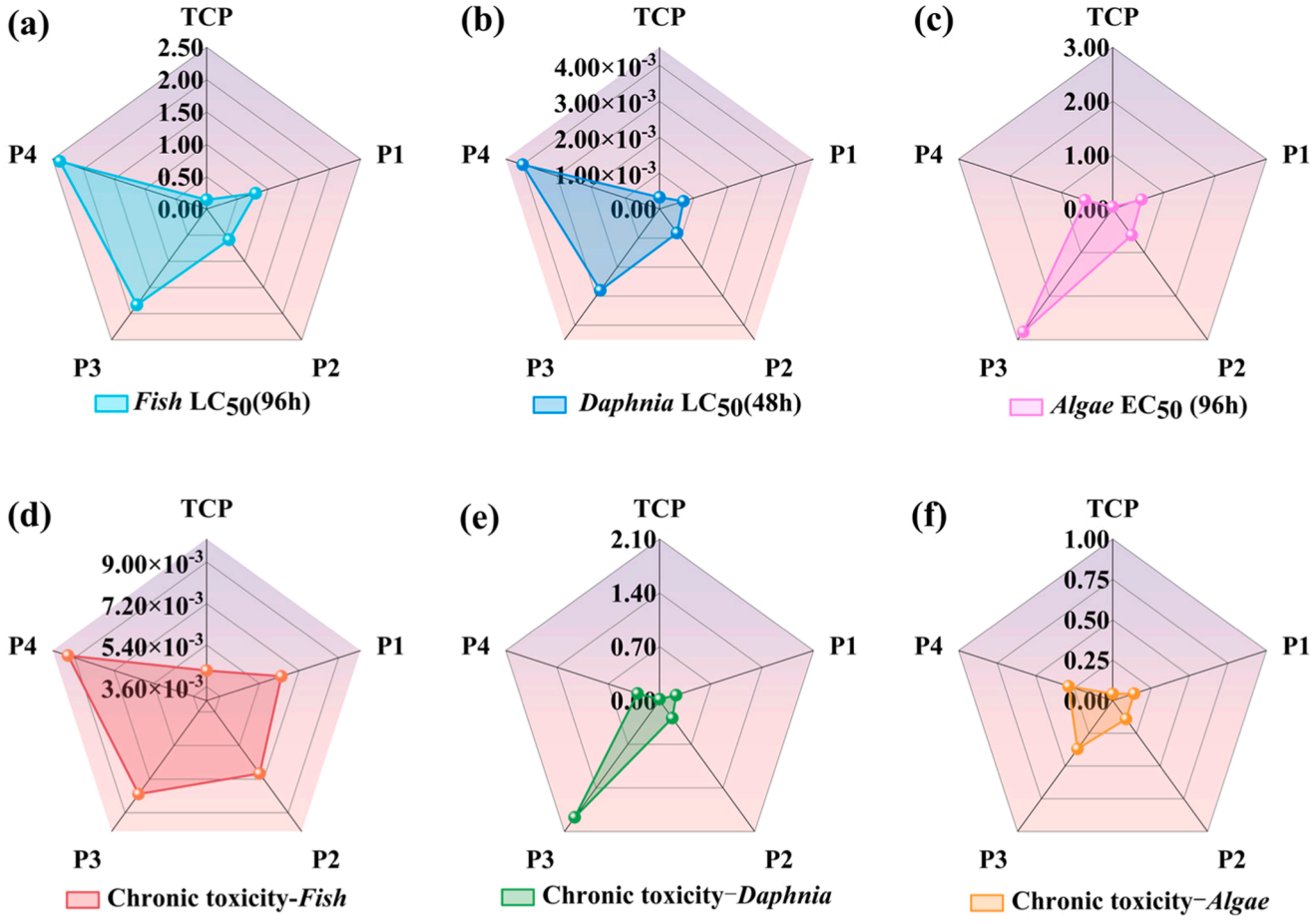

Fig. 6. Prediction of toxicity of TCP and its metabolites to fish, *daphnia* and algae using the ECOSAR model.

was 4.6 times higher than that of TCP (0.037 mg/l), and its chronic toxicity concentration to green algae (0.788 mg/L) was 19 times higher than that of TCP (0.042 mg/L).

4. Conclusion

In summary, our results suggested that *S. obliquus* have self-adaptation and detoxification in the toxicity defense process, although the algae produced significant oxidative damage and chlorophyll synthesis was affected during the first 7 d of exposure. The growth rate of algae was inhibited during the absorption and degradation process, and self-repair of algae occurred at a later stage. This phenomenon can be attributed to algal degradation and metabolism of TCP, resulting in its conversion to less toxic metabolites (P1, P2, P3, P4). Prediction of metabolite toxicity by ECOSAR software confirmed these experimental results. In cases of combined exposure, the addition of high concentration of HA (10 mg/L) could produce significant toxicity to algae under combined exposure. Moreover, ROS were still produced in algae under HA alone; in contrast, mixed nitrogen sources attenuated this toxicity. Our study emphasizes the importance of considering coexisting contaminants, different exposure concentrations of TCP, and different exposure times in ecological risk assessment, taking into account real water environment TCP exposure concentrations. Considering the laboratory limitations, future work could consider a multi-omics study to reveal the toxicity mechanisms of these chemicals at the molecular level.

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CRediT authorship contribution statement

Tianlie Luo: Writing – review & editing, Supervision, Methodology, Formal analysis. **Jingjing Shi:** Writing – original draft, Methodology, Investigation, Formal analysis. **Ping Zhang:** Writing – review & editing, Data curation. **Shuang Yang:** Writing – review & editing, Methodology. **Guo Liu:** Supervision, Methodology. **Willie J.G.M. Peijnenburg:** Supervision, Methodology, Writing – review & editing.

Declaration of competing interest

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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2025.107330.

Data availability

Data will be made available on request.

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