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Toxic effects of pesticides on the marine microalga *Skeletonema costatum* and their biological degradation

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Abstract Pesticides are considered to be toxic pollutants that are harmful to the health of humans and animals or dangerous to the environment. The pesticides chlorpyrifos, acetochlor, and dicofol, representatives of organophosphorus, acyl amide, and organochlorine pesticides, are widely used in agriculture and have been frequently detected in aquatic environments. However, the toxicity of these pesticides to marine microbes is unclear. In this study, the toxic effects of the three pesticides on a representative marine microalga, the diatom *Skeletonema costatum* (*S. costatum*), were investigated. Microalgal cell density and chlorophyll-*a* (Chl-*a*) content were analyzed to investigate the toxic effects after *S. costatum* was exposed to the three pesticides both individually and in combination. The N/P ratio in the culture medium and the degradation of the pesticides were also analyzed during exposure. Individual acute toxicity analysis indicated that the growth of *S. costatum* was significantly inhibited by acetochlor, followed by the effect of dicofol and chlorpyrifos. The cell membrane of the microalgae was impaired by acetochlor. Combined toxicity analysis indicated that the presence of acetochlor increased the toxicity of dicofol and chlorpyrifos. In contrast, the presence of dicofol reduced the toxicity of acetochlor and chlorpyrifos. The N/P ratio in the culture medium was largely increased at late growth stages; however, it was significantly lowered when the microalgae were exposed to chlorpyrifos compared to acetochlor and dicofol. The degradation of the pesticides was promoted by microalgae, suggesting that microalgae might contribute to the removal of these pesticides from the marine environment. The toxic mechanism of the pesticides on the marine microalgae and the fates of pesticides in the ocean need to be further studied.

Keywords Microalgae, Pesticide, Toxicity, Degradation, *Skeletonema costatum*

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1. Introduction

Pesticides are chemical substances that are indispensable in modern agricultural systems allowing for a noticeable increase in crop yields and food production by controlling weeds, insects, and plant diseases (Bondareva and Fedorova, 2021). The annual global amount of pesticides used is approximately 1.0×10^6–2.5×10^6 tons (Jiao et al., 2019). China is the largest agricultural country, and the average use of pesticides per hectare is about 1.5–4 times the global average (Zhang et al., 2015; Lai, 2017). Pesticides are applied for specific purposes, however, less than 1% of pesticides reach the target organisms while most are distributed in the atmosphere, soil, water bodies, and eventually enter the ocean

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(Vymazal and Březinová, 2015; Tsaboula et al., 2019). Therefore, pesticide residues have become one of the major causes of water pollution (Sun et al., 2019).

Hydrophobic pesticides such as chlorpyrifos, acetochlor, or dicofol, corresponding to organophosphorus, chloroacetamide, and organochlorine pesticides, respectively, have been used in agriculture for their high biological activity and low cost (Hu et al., 2020), and are widely distributed in aquatic environments (Xiao et al., 2017; Lei et al., 2018; Hu et al., 2020; Li et al., 2021). Acetochlor was detected in the surface water of the Biliuhe Reservoir, a drinking water sources in Dalian, China, with its highest concentration reaching 61.9 ng L^{-1} (Dong et al., 2019). Another study performed an analysis of pesticide distributions in seven typical river basins in China, and the results indicated that five pesticides including acetochlor were detected at more than 80% of the points, with average concentrations ranging from 5.62 to 225.93 ng L^{-1} (Xu et al., 2019). Dicofol is frequently detected in the water and sediments of the Jiulong River, the second largest river in the Fujian Province of China (Zheng et al., 2016). Chlorpyrifos and dicofol were not only detected in the surface seawater on the coast (Liu et al., 2018; Ivorra et al., 2019), but also in the sediments in the Western Pacific (Ge et al., 2021). These results indicate that pesticides are persistent and recalcitrant pollutants in the environment.

Although the pesticides detected in the environment may be at low levels, they can be magnified relative to the initial concentration by up to 70,000 fold through bioaccumulation and biomagnification (Kim et al., 2016), and more than 95% of the applied pesticides have the potential to impact non-target organisms such as aquatic invertebrates, amphibians, birds, beneficial insects, and pets (Affum et al., 2018). For example, chlorpyrifos can alter fish and aquatic invertebrate communities, thereby affecting biodiversity (Affum et al., 2018). Zebrafish have been used as model organisms to study the neurotoxicity of the chiral pesticide acetochlor, and the results indicated that acetochlor can affect the movement behavior of zebrafish larvae and induce neurotoxins in the early development of zebrafish (Sarangi et al., 2019). Overall, research concerning the toxicity of pesticides is mainly focused on higher organisms.

Diatoms are unicellular photosynthetic protists that constitute some of the most ubiquitous microalgae contributing enormously to global primary productivity and biogeochemical cycles in both marine and freshwater environments (Falciatore et al., 2020). However, the toxic effects of pesticides on microalgae are less studied. The microalga *Skeletonema costatum* (*S. costatum*) is a planktonic diatom and that is widely distributed in coastal waters. *S. costatum* has been used as a model microalgal species for analysis of the toxic effects of organic or inorganic pollutants, such as polystyrene particles and heavy metal Hg (Yi et al., 2019; Zhang et al., 2019; Ding et al., 2019; Zhu et al., 2020).

In this study, to investigate the toxic effects of the pesticides on marine microalgae, *S. costatum* was exposed to the three representative pesticides chlorpyrifos, dicofol, and acetochlor, individually and in combination. Microalgal cell density and chlorophyll-a (Chl-*a*) content were analyzed during the exposure experiment. The degradation of the three pesticides by microalgae was also analyzed to address the fate of pesticides in marine environment. This work forms part of a more comprehensive study concerning the toxicity of more than one pesticide and the information obtained will allow for more accurate risk assessment.

2. Materials and methods

2.1 Chemical reagents and microalgae species

The pesticides chlorpyrifos, dicofol, and acetochlor were purchased from Dr. Ehrenstorfer GmbH (Germany), and their chemical characteristics are listed in Table 1. Other chemical reagents were purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd (China). The microalga *S. costatum* was provided by the Center for Collections of Marine Bacteria and Phytoplankton in the State Key Laboratory of Marine Environmental Science, Xiamen University.

2.2 Cultivation of the microalga *S. costatum*

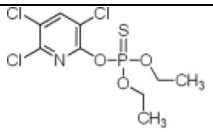
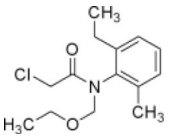
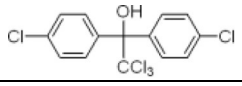
The microalga *S. costatum* was cultivated with *f*/2 medium prepared in seawater (Guillard and Ryther, 1962). The seawater was taken from the Xiamen coast, filtered twice through a 0.45 μm membrane, then sterilized and used for preparing the culture medium. The *S. costatum* at a cell density of about 5×10^4 cells mL^{-1} was inoculated into the culture medium. Cultivation was performed in triangular flasks at 20°C under 4500 Lx light irradiance with a light and dark cycle of 14 and 10 h, respectively. The flasks were shaken once a day during the culture period. Microalgal growth was monitored by analyzing the cell density (cells mL^{-1}) and content of Chl-*a* (mg L^{-1}) during the cultivation. All experiments were conducted in duplicate, and the data represent the mean values of the two replicates.

2.3 Toxic effects of three pesticides on the microalga *S. costatum*

2.3.1 Acute toxicity analysis

To test the individual acute toxic effects of the three pesticides on the microalgae, cultivation was performed in 250-mL flasks. The pesticides with final concentrations of chlorpyrifos (0.1, 0.3, 0.4, 0.5, and 0.6 mg L^{-1}), acetochlor (0.01, 0.025, 0.05, 0.1, and 0.25 mg L^{-1}), and dicofol (0.1, 0.2, 0.3, 0.4, and 0.5 mg L^{-1}) were added into flasks and

Table 1 Physicochemical properties of the three studied pesticides

Pesticide name	CAS	Chemical name	Structural formula	LogK_{ow}	Solubility in water (mg L^{-1}, 25℃)	Boiling point (℃)
Chlorpyrifos	2921-88-2	O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate		3.78	1.4	375.9
Acetochlor	34256-82-1	2'-Ethyl-6'methyl-N-(ethoxy-methyl)-2-chloroacetanilide		5.19	223	391.5
Dicofol	115-32-2	1,1-bis(4-chlorophenyl) 2,2,2 trichloroethanol		4.28	0.8	454.73

were dried under a stream of N_2 to remove methanol in the pesticide solutions, and then, culture medium was added. Solutions without pesticide served as controls. After inoculation, the cell density was analyzed at 24 h, 48 h, 72 h, and 96 h to monitor the microalgal growth.

The half effect concentration (EC_{50}) at 24 h, 48 h, 72 h, and 96 h were calculated according to the following Berkson Logit method. The cell growth rate (U_t) was calculated according to eq.(1), in which U_t is the growth rate at time t (d) (cells mL^{-1} d^{-1}); and N_t and N_0 are the numbers of cells (cells mL^{-1}) at time t and at the starting time, respectively.

$$U_t = \frac{\ln N_t - \ln N_0}{t - t_0}. \tag{1}$$

Cell growth inhibition rate I (%) was calculated according to eq.(2) (Šepič et al., 2003), in which I is the cell growth inhibition rate (%); U_c and U_t is the microalgal growth rate (cells mL^{-1} d^{-1}) of the control and treatments at time t, respectively.

$$I = \frac{U_c - U_t}{U_c} \times 100. \tag{2}$$

The S-shaped dose-response curve is converted to a straight line by the logit transformation, and the concentration effect is obtained using the eq.(3), where I_i is the cell growth inhibition rate (%) corresponding to the concentration of pesticide C_i in the treatment group i (mg L^{-1}). F test of the regression equation using $p<0.05$ indicates that the result is reliable. When the inhibition rate is 50%, the EC_{50} and the confidence interval are calculated using eq.(3).

$$\mathrm{Log}\frac{I_i}{100 - I_i} = A + B\mathrm{Log}C_i. \tag{3}$$

Next, based on individual toxicity experiments, combined acute toxicity effect was analyzed by exposing two or three pesticides simultaneously, according to the toxicity units (TUs) at a ratio of 1:1 or 1:1:1. In this study, the acute toxicity experiments of the combined effect were carried out under five TU gradients (0.05, 0.1, 0.3, 0.5, and 0.70 TU). The concentrations of the three pesticides were set as 0.0286, 0.0572, 0.172, 0.286, and 0.400 mg L^{-1} (chlorpyrifos), 0.007, 0.014, 0.042, 0.070, and 0.098 mg L^{-1} (acetochlor), and 0.0215, 0.0430, 0.129, 0.215, and 0.301 mg L^{-1} (dicofol), for 0.05, 0.10, 0.30, 0.50, and 0.70 TU, respectively. Cultivation was performed for 96 h, samples were taken every 24 h for analysis of the microalgal cell density. Experiments were conducted in duplicate. Data represent the mean values of the two replicates.

The combined acute toxic effect was evaluated by an additive index method using the eq.(4) (Marking, 1977), where S is the sum of toxic effects; and C_i and $EC_{50,i}$ (i=1, 2, 3,..., n) represent the concentration of the i-th component of the mixture at half inhibition effect and the EC_{50} of the i-th component exposed individually, respectively.

$$S = \frac{C_1}{EC_{50,1}} + \frac{C_2}{EC_{50,2}} + \frac{C_3}{EC_{50,3}} + \ldots\ldots + \frac{C_i}{EC_{50,i}},$$
$$S \leqslant 1,\ AI = \frac{1}{S} - 1.0;\ S > 1,\ AI = 1.0 - S. \tag{4}$$

The combined effects of pesticides were evaluated by measuring AI. AI>0 indicates a synergistic effect, while AI<0 indicates an antagonistic effect, and AI=0 indicates an additive effect.

2.3.2 *Chronic toxicity analysis*

Next, chronic toxicity analysis was also performed. Pikula et al. (2019) exposed marine microalgae to biodiesel fuel for 7 days to analyze the chronic toxic effect. In this study, based on the results of 96 h EC_{50} individual acute toxicity experiments, *S. costatum* was exposed to pesticides with concentrations of 0.1, 0.5, and 1.0 of the 96 h EC_{50} for 16 days (a whole growth cycle) to analyze the chronic toxic effects of the three individual pesticides. During the culture period, samples were taken daily to determine the microalgal cell density and Chl-*a* content as described above.

2.4 Degradation of the pesticides

Degradation experiments were conducted at 0.1 times the 96 h EC_{50} of each target pesticide as initial concentrations. In the individual pesticide degradation experiment, the initial concentrations of each target pesticide were 0.0572 mg L^{-1} (chlorpyrifos), 0.014 mg L^{-1} (acetochlor), and 0.0430 mg L^{-1} (dicofol). In the degradation experiment with binary combinations, the initial concentrations were 0.0237 mg L^{-1} (chlorpyrifos) and 0.0058 mg L^{-1} (acetochlor) for the combination of chlorpyrifos-acetochlor; 0.0405 mg L^{-1} (chlorpyrifos) and 0.0304 mg L^{-1} (dicofol) for the combination of chlorpyrifos-dicofol; and 0.0080 mg L^{-1} (acetochlor) and 0.0245 mg L^{-1} (dicofol) for the combination of acetochlor-dicofol. In the degradation experiment with ternary combination, the initial concentrations for chlorpyrifos, acetochlor, and dicofol were 0.0323, 0.0079, and 0.0245 mg L^{-1}, respectively. During the cultivation, the concentrations of pesticides in the culture medium were measured.

2.5 Analytical methods

2.5.1 Cell density analysis and cell morphological observation

To measure microalgal cell density, 20 μL of Logul iodine solution was added to 1 mL of culture solution to fix the microalgal cells, and the cells were counted under a microscope (Leica DME B/CA, Germany). The Logul iodine solution was prepared by dissolving 1.0 g of I_2 and 1.5 g of KI in 25 mL distilled water in a brown reagent bottle. After the microalgae were exposed to acetochlor (0.01, 0.025, 0.05, 0.1, 0.25 mg L^{-1}) for 96 h, the cellular morphology was observed under the fluorescence microscope (Axioskop40 FL, Germany).

2.5.2 Measurement of chlorophylla content

Microalgal cells from 50 mL culture solution were collected by filtration with a 0.45 μm cellulose acetate membrane. The Chl-*a* was extracted from the membrane with 10 mL of 90% acetone. Then, the content of Chl-*a* in the culture was analyzed using a spectrophotometry-based method (Brand et al., 1981).

2.5.3 GC-MS analysis of the pesticides

For analyzing the concentration of pesticides in the culture medium, 50 mL culture samples were collected from different culture time and filtered over a GF/F filter (diameter: 47 mm, pore size: 0.7 mm; precombusted at 450°C for 4 h). The filtrate was spiked with 25 μL of 5 mg L^{-1} surrogate standards (tributyl phosphate, butachlor, and Tetradifon) obtained from Dr. Ehrenstorfer GmbH (Germany), and then drawn through a C18 solid-phase extraction (SPE) column (ENVI 18, Supelco, USA). The column was dried with N_2 for 30 min and then eluted with 5 mL of methanol. The eluant was evaporated under N_2, dissolved in a 1 mL mixture (acetone-hexane =1:1, V/V), and filtered through a 0.22 μm nylon filter.

The pesticide concentrations were determined by GC-MS (Agilent 7890-5975B, Agilent, USA) equipped with a DB-5MS capillary column (50 m×0.25 mm, 0.25 μm). The oven temperature was initially held at 80°C for 2 min and was then increased at 10°C min^{-1} to 166°C for 1 min, 2°C min^{-1} to 178°C for 4 min, 2°C min^{-1} to 186°C for 5 min, 3°C min^{-1} to 230°C for 7 min, and finally increased at 8°C min^{-1} to 300°C for 20 min. The corresponding temperatures of the transport line and ionization source were 300°C and 285°C, respectively. The carrier gas was high-purity He with a flow rate of 1.0 mL min^{-1}; the injection port temperature was 260°C, and 2.0 μL sample was injected.

2.5.4 Analysis of active phosphate and nitrogen in the culture medium

The active phosphate and nitrate/nitrite nitrogen were analyzed during the cultivation. Active phosphate was analyzed according to the method described by Ohashi (Ohashi et al., 1978). Nitrate/nitrite was measured using a flow injection analysis method (Johnson and Petty, 1983).

3. Results and discussion

The growth of *S. costatum* was monitored by determining the cell density and Chl-*a* content. The growth curves based on the cell density and Chl-*a* content were similar (Figure 1). Both the cell density and the content of Chl-*a* increased after inoculation, without a significant slow growth period; this may be due to the good growth conditions for the microalgae species, the high initial cell density (5×10^4 cells mL^{-1}), or sufficient nutrients in the culture medium. The microalgal cell density reached the maximum on the 12th day at about 1.33×10^6 cells mL^{-1}, while the maximum content of Chl-*a* reached about 1.25 mg L^{-1} on the 14th day, slightly later than that of the maximum cell density, and then, the growth of *S. costatum* decreased rapidly. Toxic effects of pesticides on *S. costatum* were analyzed by monitoring the cell density and Chl-*a* content after exposure to the pesticides.

3.1 Individual toxic effects of the three pesticides on *S. costatum*

3.1.1 Acute toxicity effect

For the acute toxicity analysis, *S. costatum* was exposed individually to chlorpyrifos, acetochlor, and dicofol at different concentrations, and the cell density was analyzed for the first 96 h after inoculation. The EC_{50} values at 24 h, 48 h,

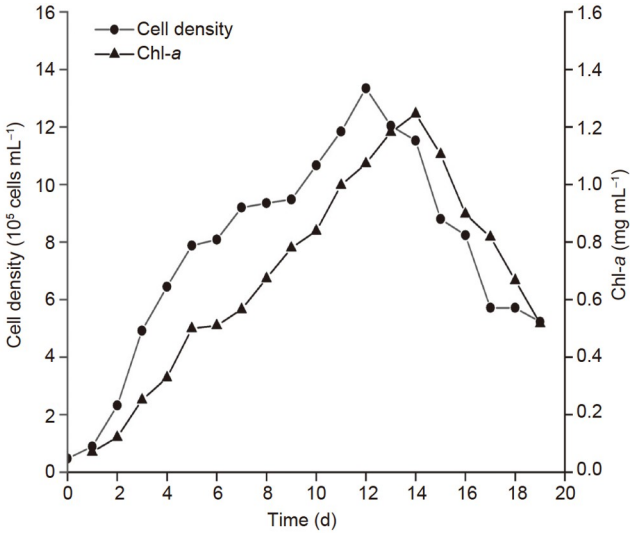


Figure 1 Growth curves of *S. costatum*.

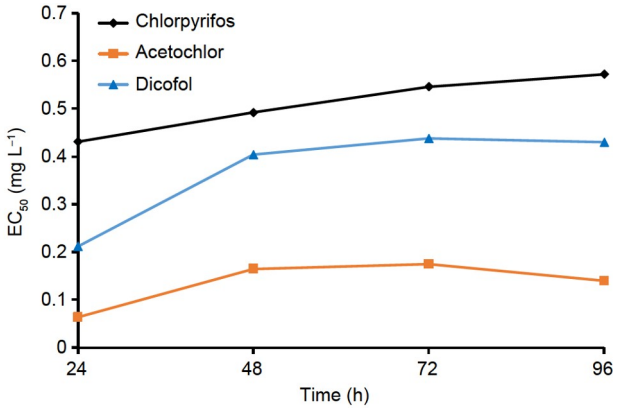


Figure 2 Acute toxicity of the three pesticides to the microalga *S. costatum*.

72 h, and 96 h were calculated according to the growth curve of *S. costatum*. As shown in Figure 2, the EC_{50} of chlorpyrifos for *S. costatum* gradually increased with time, indicating that the toxic effect gradually decreased with time. The EC_{50} values of acetochlor and dicofol for *S. costatum* increased from 24 h to 72 h and then slightly decreased, indicating that the toxic effects of acetochlor and dicofol tended to decrease initially and then increase. The EC_{50} values were significantly different for the three pesticides at different times ($p<0.05$) and shown same trends: chlorpyrifos>dicofol>acetochlor. The result indicated that *S. costatum* was most sensitive to acetochlor and had the strongest tolerance to chlorpyrifos.

The 96 h EC_{50} values of chlorpyrifos, acetochlor, and dicofol were 0.572, 0.14, and 0.430 mg L^{-1}, respectively. According to the toxicity classification standards of pesticides to algae, those with a 96 h EC_{50} lower than 0.3 mg L^{-1} are classified as highly toxic pesticides, and those with a 96 h EC_{50} between 0.3 and 3 mg L^{-1} are classified as middle toxic pesticides (Zhou et al., 1996; Reyes et al., 1999). Acetochlor was identified as highly toxic to *S. costatum*, while chlorpyrifos and dicofol were moderately toxic pesticides.

3.1.2 *Chronic toxicity effect*

To analyze the chronic toxic effects of the pesticides, *S. costatum* was exposed to 0.1, 0.5, and 1.0 times of the 96 h EC_{50} values of the three pesticides for a whole growth cycle (16 days). Cell density and Chl-*a* content were analyzed daily (Figure 3). In the chlorpyrifos treatment, 0.0572, 0.286, and 0.572 mg L^{-1} were amended (Figure 3a). The cell density was not significantly different in the 0.0572 mg L^{-1} treatment from that of the control during the initial 12 days of cultivation, and it was slightly lower than that of the control after 12 days. In the 0.286 mg L^{-1} treatment, the cell density was lower than that of the control during the first 6 days and then was significantly higher than that of the control during the later cultivation period. The cell density in the 0.572 mg L^{-1} treatment was lower than that of the control during the first 12 days of culture and was significantly higher than that of the control during the later growth period. This result indicated that the toxicity of chlorpyrifos to *S. costatum* decreased with culture time.

In the acetochlor treatment, 0.014, 0.07, and 0.14 mg L^{-1} acetochlor were supplemented in the culture medium. There was no significant difference in the cell density between the 0.014 mg L^{-1} treatment and the control, while the cell densities in the 0.07 mg L^{-1} and 0.14 mg L^{-1} treatments were lower than that in the control. The growth period was shortened as the concentration of acetochlor increased (Figure 3b). In the dicofol treatment, 0.043, 0.215, and 0.43 mg L^{-1} were employed. Similar to the acetochlor treatment, the growth impairment by dicofol occurred at a high concentration (0.43 mg L^{-1}) (Figure 3c).

The effects of the three pesticides on the Chl-*a* content were similar to those for the cell density. When 0.1 times of the 96 h EC_{50} value for the three pesticides was supplemented, Chl-*a* content was not significantly different from that of the control. However, the synthesis of Chl-*a* was almost completely inhibited when the microalgae were exposed to acetochlor and dicofol at 1.0 times of the 96 h EC_{50} value. We noticed that the Chl-*a* content in the chlorpyrifos treatment of 1.0 times the 96 h EC_{50} value was higher than that in the control at the later growth phase. These results again indicated that the toxic effects of chlorpyrifos to *S. costatum* can be ameliorated, but those of acetochlor and dicofol are irreversible.

Among the three pesticides, acetochlor, one of the most widely used herbicides, showed the strongest toxic effect on microalgal growth. The effects of the pesticide on the cell morphology of *S. costatum* were observed after exposure to different concentrations of acetochlor

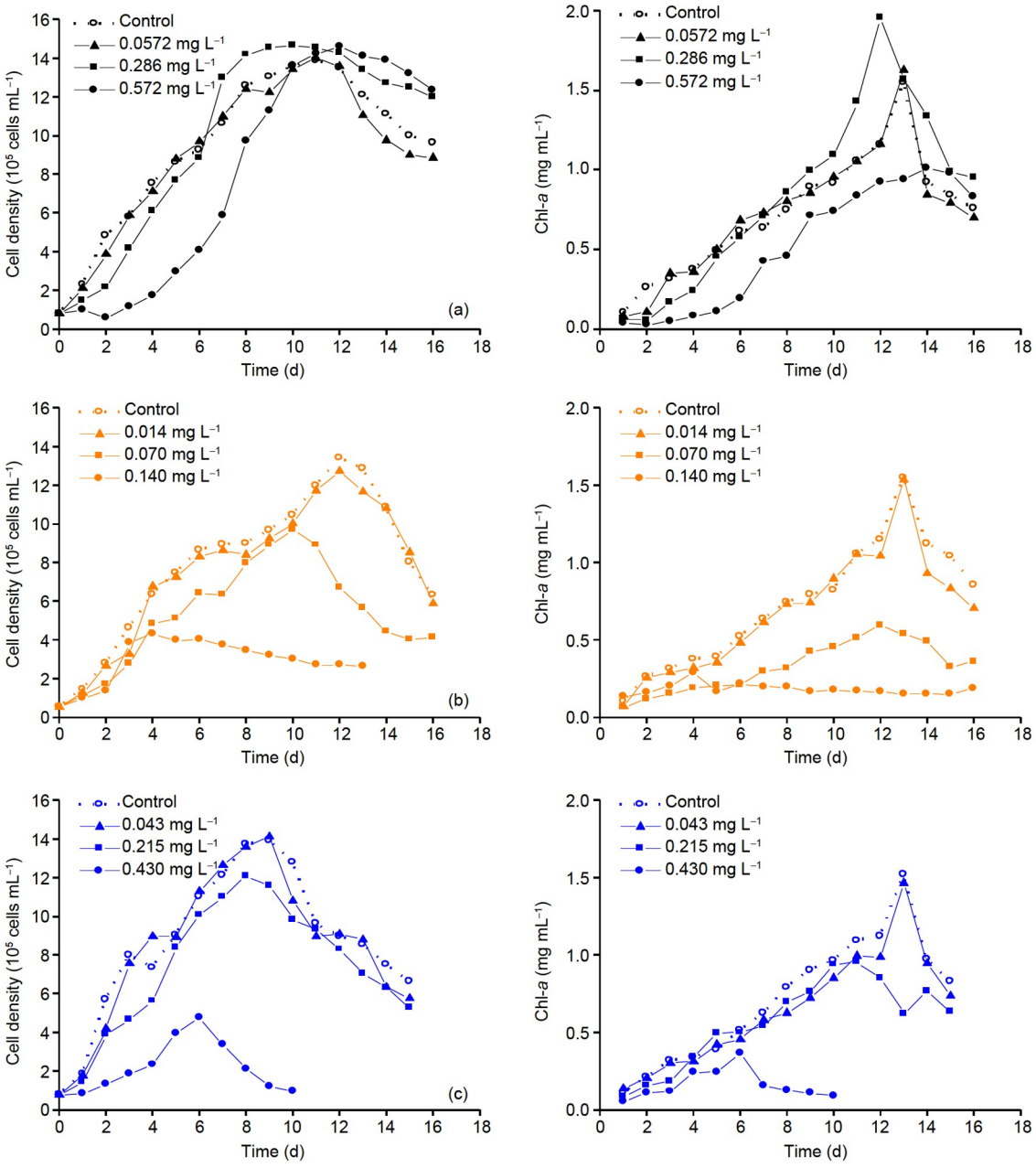


Figure 3 Chronic toxic effects of individual pesticides on *S. costatum*. The microalga *S. costatum* was exposed to 0.1, 0.5 and 1.0 times of 96 h EC50 of chlorpyrifos (a), acetochlor (b) and dicofol (c).

(0.01–0.25 mg L^{-1}) for 96 h (Figure 4). As shown in Figure 4c, some of the cells showed abnormal cell division and morphology. When *S. costatum* was exposed to 0.25 mg L^{-1} acetochlor for 96 h, the microalgal cell shape disappeared completely and became a 'cluster' (Figure 4e), indicating that the cell membranes of the microalgae were damaged by acetochlor. The toxicity of acetochlor to the green alga *Scenedesmus vacuolatus* has also been observed (Junghans et al., 2003). It was observed that the growth of *S. vacuolatus* was impaired by acetochlor alone at the EC_{50} value of 0.12 mg L^{-1}, and was completely inhibited by exposure to mixtures of acetochlor and seven other herbicides (Junghans et al., 2003). Other studies indicated that pesticides can destroy the cell membranes of algae, making it easier for pesticide molecules to enter the cell and interfere with or disrupt life processes such as photosynthesis, respiration, and cell division (Stratton, 1989; Karen et al., 2000). A recent study reported that herbicides showed toxicity to phytoplankton at environmental concentrations, and they can disturb multiple metabolic pathways related to photosynthesis and carbon metabolism of diatoms, including the Calvin cycle, the tricarboxylic acid (TCA) cycle, and glycolysis/gluconeogenesis (Yang et al., 2019).

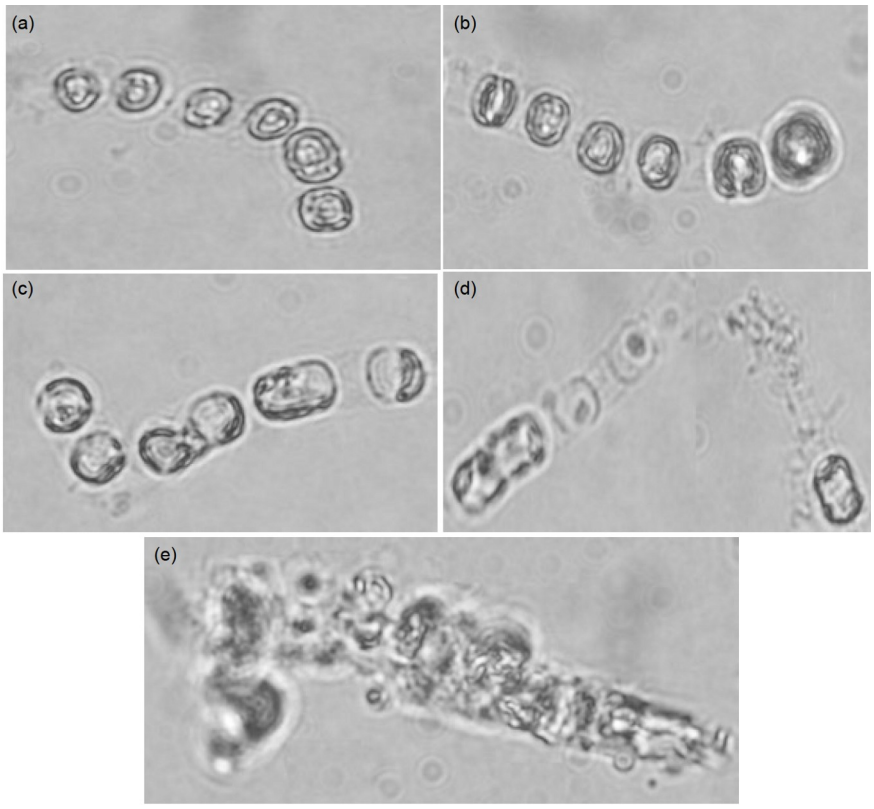


Figure 4 Effect of acetochlor on *S. costatum* cell morphology. (a)–(e) The microalgae were exposed to 0, 0.01, 0.025, 0.1 and 0.25 mg L^{-1} acetochlor for 96 h, respectively. The cells were observed under a fluorescence microscope with 100× magnification.

3.2 Joint toxic effects of the three pesticides on *S. costatum*

Since pesticides are usually detected as mixtures in the environment, next the joint toxic effects of the three pesticides on *S. costatum* were investigated. The *S. costatum* was exposed to two or three pesticides under five TU gradients. The results were similar to the results with individual exposure (Table 2). The EC_{50} values were significantly different within the exposure times under the four combination treatments ($p<0.05$). The EC_{50} values were the lowest in the 24 h exposure, and 96 h EC_{50} values were 2.2–3.9 times higher than 24 h EC_{50} values, indicating that the toxicities of the four pesticide combinations were all weakened by prolonging exposure (Table 2).

The additive index method was used to evaluate the joint toxicity (Marking, 1977). The four combinations of the pesticides all showed synergistic effect at 24 h (Table 2). Chlorpyrifos-acetochlor showed a synergistic effect for *S. costatum* at 24 h, 48 h, 72 h, and 96 h. Both chlorpyrifos-dicofol and acetochlor-dicofol had synergistic effects at 24 h but had antagonistic effects at 48 h, 72 h, and 96 h. In the ternary combined exposure treatment, there was a synergistic effect at 24 h and 48 h but an antagonistic effect at 72 h and 96 h.

Under the individual exposure, the toxicity of the three pesticides to *S. costatum* was in the order: acetochlor > dicofol > chlorpyrifos. Under the combined exposure, all the binary and ternary combinations at low concentrations showed a synergistic effect at 24 h, indicating that there was an irritability reaction in the microalgae. Under the longer exposure times (72 h and 96 h), acetochlor-chlorpyrifos showed a synergistic effect, while dicofol-acetochlor and dicofol-chlorpyrifos showed antagonistic effects. The results suggest that the presence of dicofol reduced the toxicities of acetochlor and chlorpyrifos. In the ternary combination, 24 h and 48 h showed synergy, but 72 h and 96 h showed antagonism, suggesting that the reduction of toxicity by dicofol was weaker than the synergy between acetochlor and chlorpyrifos in a short period of time but was stronger after a longer time. The result indicated that the synergistic or antagonistic joint effects of pesticides on algae depend on the duration and magnitude of exposure. Other studies also found such a phenomenon. For example, joint action of two herbicides (Mikado and Viper) on the growth of algae *Pseudokirchneriella subcapitata* showed antagonistic effects at lower mixture concentrations, while at higher doses, their interaction induced synergistic effects (Marques et al., 2012). However, another study investigated the interactions between herbicides and organophosphorus insecticides, including chlorpyrifos, on *P. subcapitata*, but none of the organophosphorus insecticides tested was found to enhance

Table 2 Evaluation of joint toxicity of the three pesticides on *S. costatum* [a)]

Pesticide combination		Time			
		24 h	48 h	72 h	96 h
Chlorpyrifos-acetochlor	EC_{50}*	0.185	0.266	0.353	0.414
	AI	1.703	0.88	1.833	0.208
	Interaction	S	S	S	S
Chlorpyrifos-dicofol	EC_{50}*	0.145	0.509	0.658	0.57
	AI	2.448	–0.018	–0.316	–0.14
	Interaction	S	A	A	A
Acetochlor-dicofol	EC_{50}*	0.271	0.684	0.614	0.708
	AI	0.845	–0.368	–0.228	–0.416
	Interaction	S	A	A	A
Chlorpyrifos-dicofol-acetochlor	EC_{50}*	0.223	0.299	0.405	0.564
	AI	0.495	0.115	–0.215	-0.692
	Interaction	S	S	A	A

a) EC_{50}* is the TU value corresponding to the semi-inhibitory effect under the combined exposure condition. A: Antagonism; S: Synergy.

the effect of the herbicides (Munkegaard et al., 2008). These results suggest that the joint toxicity effects are very complex and may vary by pesticide type, concentrations of pesticides, exposure times, and organisms used. It has been suggested that the effect of one chemical being synergized or antagonized by the presence of another may due to change of uptake, activity, degradation, or biotransformation processes (Cedergreen and Streibig, 2005). The synergistic mechanism between acetochlor and chlorpyrifos and the antagonistic mechanism of dicofol to acetochlor and chlorpyrifos need to be further addressed.

The joint chronic toxicity of the three pesticides on *S. costatum* exposed to 0.1, 0.5, and 1.0 times the 96 h EC_{50} was also analyzed during the entire growth stage. The cell density of *S. costatum* gradually decreased with the increase of pesticide concentrations in the binary and ternary combination treatments (data not shown). The growth rate under 0.1 times the 96 h EC_{50} was not significantly different from that of the control; that under 0.5 and 1.0 times the 96 h EC_{50} exposure significantly inhibited the growth of *S. costatum*. Overall, the joint toxicity was increased by the presence of the herbicide acetochlor in both binary and ternary treatments, leading to the conclusion that acetochlor is highly toxic to the microalga. The toxic mechanisms of the three pesticides individually or in combination need to be further investigated. In addition, large sensitivity differences toward herbicides have been observed within algal species (Marques et al., 2012). For example, a recent study indicated that triazine herbicide significantly altered the phytoplankton community structure in coastal waters, with the dominant group shifting from diatoms to dinoflagellates due to the higher atrazine sensitivity of diatoms compared to facultative dinoflagellates (Yang et al., 2021). The combined toxic effects of various pesticides on natural microbe communities in the marine environment under long-term exposure need to be investigated.

3.3 Changes of N/P ratio throughout exposure

The growth of phytoplankton is regulated by inorganic nutrients. Marine phytoplankton uptake of inorganic nitrogen and phosphorus basically follows the Redfield ratio of 16:1, which is often used to evaluate the conditions for phytoplankton growth (Redfield, 1958). In order to understand the effects of inorganic nutrients on the growth of *S. costatum* with individual pesticide exposure, the N/P ratio in the culture medium was analyzed during the cultivation, in the presence of 0.1 times the 96 h EC_{50} of the pesticides, since in those conditions the growth of the microalgae was almost unaffected (Figure 3). The culture inoculated with the microalgae but not exposed to the pesticides served as a control (Table 3).

As shown in Table 3, in the cultures without the microalgae, the N/P ratios in the acetochlor and dicofol treatments were similar and did not change significantly; however, the N/P ratio in the culture with chlorpyrifos, an organophosphorus pesticide, it was decreased significantly (43%, $p<0.05$) during the cultivation, suggesting that non biological degradation of chlorpyrifos occurred. The N/P ratio in all treatments increased rapidly at the late exponential growth phase (from 7th to 14th days), consistent with the rapid microalgal growth observed during this period (Figure 2). After 14 days cultivation, the growth of the microalgae declined sharply, suggesting that the declined growth of the microalgae may be partly due to the limitation of P (Zhang et al., 2012). We also observed that the N/P ratio in the middle and late stages (7th and 10th days) in the culture supplemented with chlorpyrifos was significantly lower than that

Table 3 N/P ratio in microalgal media for different incubation time periods [a)]

Pesticide treatments	Pesticide Conc. (mg L^{-1})	Microalgae	N/P					
			0 d	1 d	4 d	7 d	10 d	14 d
Chlorpyrifos	0.057	−	28.74	28.58	27.87	25.39	19.22	16.31
		+	28.74	23.27	41.44	44.53	237.23	765.06
Acetochlor	0.014	−	28.74	27.31	26.75	27.16	28.02	26.80
		+	28.74	19.34	39.18	211.86	267.94	874.60
Dicofol	0.043	−	28.74	28.67	29.01	28.73	26.96	28.10
		+	28.74	17.91	37.56	264.90	414.97	885.60
Control	0	+	28.74	19.26	41.20	217.49	339.27	950.13

a) + and – indicate the culture with and without the microalgae inoculated, respectively.

with acetochlor and dicofol as well as the control without pesticides. Therefore, it is possible that the chlorpyrifos was degraded or converted to active phosphorus to provide P nutrients for the microalgae. The result may also explain that shown in Figure 3, where the cell density decline rate in the later stage was significantly lower than those under dicofol and acetochlor exposure.

3.4 Degradation of pesticides during the cultivation

The concentrations of the pesticides in the culture medium were analyzed during the cultivation. The degradation rate constants (k) in the presence of *S. costatum* were 3.1–4.5 times higher than those in the control without algae, indicating that the presence of microalgae in the culture significantly promoted the degradation of the target pesticide (Table 4). The half-lives of chlorpyrifos, acetochlor, and dicofol in the control without the microalgae were 5.40, 42.27, and 18.10 days, respectively, being decreased to 1.23, 10.06, and 5.92 days by the addition of the microalgae. About 67–77% of the pesticides were degraded in the culture system under individual exposure conditions. Among the three pesticides, the degradation of acetochlor was the slowest, followed by dicofol and then chlorpyrifos, where the result consistent with the toxicity order. The result suggests that the high toxicity of the pesticides may partly due to their resistance to biological degradation.

The degradation of the three pesticides under combined exposure was also analyzed (Table 4). The degradation rate of chlorpyrifos was not affected by the presence of dicofol, slightly decreased when combined with acetochlor, and was the lowest when the three pesticides co-existed. The degradation rate of acetochlor was increased when chlorpyrifos and dicofol were present, and was increased by 3.4 times when dicofol was co-exposed. In contrast, the degradation of dicofol was inhibited by the presence of both chlorpyrifos and acetochlor (Table 4). These results may partly explain the antagonistic effect of dicofol with chlorpyrifos and acetochlor (Table 2).

The degradation of pesticides was dominated by biodegradation, which accounted for about 37–77% of the degradation under individual or combined exposure conditions. A similar result was obtained in the study of the stability of atrazine herbicide in seawater, and it was found that microbes were the key factors leading to the degradation of the herbicide (Yang et al., 2021). In the control without inoculation of the microalgae, decreased concentrations of the three pesticides were observed, suggesting that in addition to the biodegradation, other processes such as chemical hydrolysis and photodegradation may have contributed to the degradation of the pesticides (Yuan et al., 2018). Therefore, biodegradation, chemical hydrolysis and photodegradation may be involve in the removal of the pesticides from environment. The results were consistent with the observations regarding the changes in the N/P ratio under single chlorpyrifos exposure (Table 3). These results suggested that there were multiple degradation pathways for pesticides during the cultivation.

Among the three pesticides, acetochlor had relatively longer half-lives under both individual and combined conditions. Recently, degradation of acetochlor by soil was investigated and it was found that 10 degradation products and eight acetochlor conjugates were generated through dealkylation, hydroxylation, and thiol conjugation, glycosylation pathways were also identified (Zhang et al., 2021). Chlorpyrifos, one of the most widely used organophosphorus insecticides, is commonly degraded to 3,5,6-trichloro-2-pyridinol (TCP), which is water-soluble, toxic, and has a longer half-life in the environment (65−360 days) than chlorpyrifos (Fang et al., 2019). Some specific microorganisms including bacteria and fungi that are capable of degrading chlorpyrifos or TCP, have been isolated from soil (Zhang et al., 2017; Fang et al., 2019). A bacterial strain *Cupriavidus nantongensis* X1 could efficiently degrade both chlorpyrifos and TCP to 3,6-dihydroxy pyridine-2,5-dione (Fang et al., 2019). The degradation of chlorpyrifos and dicofol by a white-rot fungus *Trametes versicolor* was reported (Hu et al., 2020). Two compounds, O,O-diethyl thiophosphate and diethyl phosphate, were detected as transformation products of chlorpyrifos, whereas dicofol was degraded

Table 4 Degradation kinetic parameters of pesticides under different conditions[a)]

Treatment	Pesticides	Microalgae	Degradation rate constant k	$T_{1/2}$ (d)	Contribution (%)
Single expose	Chlorpyrifos	−	0.1285	5.4	/
		+	0.5619	1.23	77.1
	Acetochlor	−	0.0164	42.27	/
		+	0.0689	10.06	76.2
	Dicofol	−	0.0383	18.1	/
		+	0.117	5.92	67.3
Chlorpyrifos-Acetochlor	Chlorpyrifos	−	0.178	3.89	/
		+	0.4119	1.68	56.8
	Acetochlor	−	0.029	23.9	/
		+	0.1292	5.36	77.6
Chlorpyrifos-Dicofol	Chlorpyrifos	−	0.1288	5.38	/
		+	0.5697	1.22	77.4
	Dicofol	−	0.0791	8.76	/
		+	0.1258	5.51	37.1
Acetochlor-Dicofol	Acetochlor	−	0.0626	11.07	/
		+	0.2337	2.96	73.2
	Dicofol	−	0.0424	16.34	/
		+	0.0899	7.71	52.8
Chlorpyrifos-Acetochlor-Dicofol	Chlorpyrifos	−	0.0903	7.67	/
		+	0.3594	1.93	74.9
	Acetochlor	−	0.0372	18.63	/
		+	0.1247	5.56	70.2
	Dicofol	−	0.0509	13.61	/
		+	0.1613	4.3	68.4

a) + and – indicate the culture inoculated with and without the microalgae, respectively.

to benzaldehyde (Hu et al., 2020). Recently, O,O-diethyl thiophosphate was also identified as a transformation product of chlorpyrifos by microalgal samples composed mainly of *Chlorella* sp. and *Scenedesmus* sp. (Avila et al., 2021). The degradation pathways in the microalga *S. costatum* and the toxicity of their degradation products to marine microbes need to be further investigated. We noticed that the transformation products of these pesticides showed a carboxyl-rich alicyclic molecule (CRAM)-like structure (Fang et al., 2019; Zhang et al., 2021) that was proposed as representative of the oldest, most refractory forms of DOM (Lechtenfeld et al., 2015). This suggests that refractory molecules accumulated during the degradation of pesticides have contributed to the microbial carbon sequestration (Jiao et al., 2010). Indeed, a recent study indicated that herbicides can affect the composition of chromophoric dissolved organic matter (CDOM) produced by diatoms, thus may influence the carbon sequestration potential in the coastal seawater (Yang and Zhang, 2020). To evaluate the ecological risk and the fates of pesticides in the ocean, molecular processes and mechanisms of pesticides degradation and transformation by marine microbes are need to be further investigated.

4. Conclusions

The toxic effects of three widely used pesticides on the marine microalga *S. costatum* are different. Among these pesticides, acetochlor showed the strongest toxic effect, while chlorpyrifos had the weakest effect. Combined toxicity analysis indicates that the presence of acetochlor increases the toxicity of dicofol and chlorpyrifos, while the toxicity of acetochlor and chlorpyrifos could be reduced by the presence of dicofol. The pesticides were partially degraded by marine microalgae during the cultivation. This study provided new insights into the toxicity of three pesticides to marine microalgae as well as the evidence concerning the contribution of microalgae in the removal of these pesticides from the environment. The molecular processes and mechanisms of degradation of pesticides by marine microalgae need to be further investigated.

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