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# Toxicity of Chlorpyrifos and Dimethoate to the Ciliate *Urostyla grandis*, with Notes on Their Effects on Cell Ultrastructure

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**Abstract** Chlorpyrifos and dimethoate are overused agricultural pesticides that can trigger trophic cascades, resulting in toxicity to both terrestrial and aquatic organisms as well as altered ecosystems. In previous studies, substantial attention has been given to the effects of pesticides on vertebrate species and, to a lesser extent, species of zooplankton. The present study was designed to show that the fission time effective concentration in ciliates is a potential aquatic detection index for environmental monitoring. The ciliate *Urostyla grandis* was treated with doses of chlorpyrifos and dimethoate. After exposed to the pesticides, the $LC_{50}$ (*i.e.*, concentration that killed 50% of the ciliate cells within 24 h) values were 0.029 mg $L^{-1}$ for chlorpyrifos and 0.0685 mg $L^{-1}$ for dimethoate. The fission time effective concentrations after 168 h of exposure were 0.0075–0.0093 mg $L^{-1}$ for chlorpyrifos and 0.2640–0.2750 mg $L^{-1}$ for dimethoate. These results show that the fission time effective concentration is lower than the $LC_{50}$ value in ciliates, indicating that fission time effective concentration is more suitable than the $LC_{50}$ value for environmental monitoring using ciliates. The effects of chlorpyrifos and dimethoate on ciliate cell ultrastructures included agglutination of chromatin in the macronucleus, protruded and discontinuous macronuclear and micronuclear membranes, loss of integrity of mitochondrial membranes and contents, and abscission and deformation of the adoral zone of membranelles.

**Key words** ciliate; fission time effective concentration; $LC_{50}$; pesticide; ultrastructure

## 1 Introduction

It is widely known that agricultural pesticides pollute the environment and cause a series of ecological changes in natural aquatic communities (Bartha *et al*., 1965; Wheeler, 2002; Weston *et al*., 2004). More than 10000 chemicals are used for industrial and agricultural purposes (Katsumata *et al*., 2006), including organophosphate insecticides which contaminate surface water through the unintentional drift of aerial spraying during agricultural usage, watershed drainage, or accidental spillage, resulting in toxicity to aquatic organisms, land animals, and humans (Lundebye *et al*., 1997; Sabater and Carrasco, 2001; Dogan *et al*., 2011; Du *et al*., 2015). The organophosphates chlorpyrifos (O, O 0-diethyl-O-3, 5, 6-trichloro-2-pyridyl phospho-rothionate; CPF) and dimethoate (O, O-dimethyl S-methylcarbamoylmethyl phos-phorodithioate) are widely used to restrict insect pests in a range of commercial crops (Sørensen *et al*., 1995; Cho *et al*., 2002; Choung *et al*., 2013). Several reports have described the detrimental effects of chlorpyrifos and dimethoate on aquatic organisms. Chlorpyrifos has been tested in combination with atrazine and cadmium to increase the understanding of their toxicological interactions with earthworms in soil (Yang *et al*., 2015). A study on tropical reef fish reported that fish skeletal muscle is sensitive to chlorpyrifos, suggesting that muscle tissue could be a marker of chlorpyrifos exposure (Botté *et al*., 2012). Manjunatha and Philip (2015) have noted that vitellogenin levels increase in male zebrafish exposed to chlorpyrifos. Dogan *et al*. (2011) reported the clastogenic effects on liver and brain cells of rainbow trout exposed to dimethoate. Studies on zooplankton have similarly revealed a multitude of physiological and behavioral effects caused by chlorpyrifos and dimethoate. For example, dimethoate exposure significantly inhibits the swimming of the freshwater rotifers *Brachionus calyciflorus* and *Asplanchna brightwelli* (Chen *et al*., 2014), and the exposure to chlorpyrifos results in changes of zooplankton generation time and community structure.

Ciliates are a group of microeukaryotes that inhabit in terrestrial, freshwater, and marine, environments where there is sufficient water for them to move and feed. Ciliates play a key role in the microbial loop that is responsible for rapid remineralization of organic matter in the water column and in the transfer of materials and energy

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to higher organisms (Jonsson, 1986). In addition, their small size, short lifecycle, genetically homogeneous progeny, and ease of maintenance in the laboratory make them a potentially suitable model organism to evaluate the effects of contaminants on aquatic communities (Kim *et al*., 2014). In recent years, ciliates have been widely used to evaluate the effects of hypoxia (Rocke and Liu, 2014), herbicides (Bamdad *et al*., 2000), and heavy metals (Gomiero *et al*., 2013; Kim *et al*., 2014). These studies suggest that ciliates are more sensitive than multicellular organisms to environmental stresses (Gutiérrez *et al*., 2003; Dayeh *et al*., 2005). However, little is known about the morphological and biochemical changes induced in ciliates by exposure to contaminants (Martín-González *et al.*, 2005). Previous studies have considered only a few ciliate species, particularly those included in the genus *Tetrahymena* (Pyne *et al.*, 1983; Nilsson, 1997). In recent years, some studies have reported other groups, such as *Architricha indica* and *Australocirrus* cf. *australis* for cell structure analyses (extrusomes and cysts) (Li *et al*., 2014; Zhang *et al*., 2014).

Hypotrichs are the most complex and highly differentiated group of ciliates, with highly diverse cortical structures, nuclear apparatus, and special nuclear dualism. They are important in the field of cell biology; thus, we chose ciliates as the experimental subjects (Singh and Kamra, 2015; Luo *et al.*, 2017). No data about ultrastructural changes caused by pesticides have been published on spirotrichean ciliated protozoa. The concentration and conditions of exposure are the main factors determining the toxic effect of a given compound on an organism in a toxicity test (Ottoboni, 1991). Oliveira *et al.* (2011) considered the mortality interval as the minimum lethal concentration of ticks to evaluate the toxicity of the acaricide fipronil. In addition, the median lethal concentration $LC_{50}$ is widely used for measuring the toxicity of a substance in vertebrates and invertebrates (Madoni *et al*., 2000; Huertas *et al*., 2002; Yang *et al*., 2003; Ugine *et al*., 2005). However, the present study attempted to determine whether the fission time effective concentration is suitable for ciliates as an indicator of water quality. An alternative method that would be quicker and less expensive might be to use cellular observations of ciliates to determine the fission time effective concentration as a 'minimum effective concentration' of a toxic compound as an aquatic detection index.

In this study, we used the freshwater spirotrichean ciliate *Urostyla grandis* as a eukaryotic model organism to measure the toxicity of chlorpyrifos and dimethoate. *U. grandis* is a typical benthic ciliate (Madoni and Zangrossi, 2005) that reflects the conditions on the bottom of the water column; thus, it is a candidate organism for benthic aquatic monitoring. We exposed *U. grandis* to chlorpyrifos and dimethoate with different concentrations to determine the fission time effective concentration and to compare this value with $LC_{50}$. The fission time effective concentration method has been more useful than $LC_{50}$ in studies related to ciliates. After the pesticide treatment, we observed cell ultrastructural changes by transmission electron microscopy (TEM) to determine the feasibility of using fission time effective concentration as an index of pollutant monitoring in ciliates.

## 2 Materials and Methods

### 2.1 Species Test and Toxicity Bioassays

The freshwater ciliate *U. grandis* was collected from Songhua River in Harbin, Heilongjiang Province, China. A single cell was picked from the samples with a glass straw, washed repeatedly in drops of mineral water and then put into a 90-mm diameter Petri dish for culturing. The culture medium was comprised of boiled wheat grain as food sources and mineral water. The selected species was grown at room temperature (20℃). Individuals from populations reaching log-phase growth were used in the experiments. Aqueous stock solutions of 10% chlorpyrifos and 10% dimethoate were prepared. The stock solutions were diluted in sterile distilled water to obtain chlorpyrifos with the nominal concentrations of 0.0212, 0.0244, 0.0276, 0.0308, 0.0340, and 0.0372 mg $L^{-1}$; and dimethoate with the nominal concentrations of 0.50, 0.55, 0.60, 0.65, 0.70, and 0.75 mg $L^{-1}$. These concentrations were selected following previous assays in which cells were exposed to a wider range of chlorpyrifos and dimethoate. Tissue culture plates (24-well) were employed with about 100 ciliate cells per well. A control treatment was incubated in the same culture medium without any pesticide treatment. All assays were carried out in triplicate. Results of the replicates for each concentration were averaged.

To evaluate the median-lethal concentration of chlorpyrifos and dimethoate on *U. grandis*, the number of dead cells in each well after a 24-h incubation was enumerated by direct observation at low magnification. Propidium iodide was used to detect cell mortality and to quantify dead cells by flow cytometry. The median lethal concentrations (24-h $LC_{50}$) were determined using the probit method, and the goodness of fit probability was $>0.95$ at all tested concentrations.

The fission time effective concentrations of chlorpyrifos and dimethoate during cell division were investigated after 24-, 48-, 96-, 120-, 144- and 168-h exposures using fission time measurements. The dividing cells were observed under a microscope. After calculating the division times of *U. grandis*, the approximate concentration was ascertained, and the concentration range was determined using an arithmetic gradient concentration.

### 2.2 Statistical Analysis

The results obtained for the $LC_{50}$ analysis of the ciliates were compared with a probit analysis using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Based on the relationship between the chlorpyrifos and dimethoate concentrations and the ciliates, the $LC_{50}$, 95% confidence interval, and slope of the regression were calculated. Levene's test and the *t*-test were used to verify the fission time effective concentration of chlorpyrifos and dimethoate over the 168-h treatment period.

### 2.3 Electron Microscopy Techniques

After 24-h with or without a toxicant (fission time ef-

fective concentration) treatment, samples were processed as follows before the TEM observations. Specimens were prepared for TEM following the method of Nakamura *et al*. (2006). Briefly, cells (including control cells and cells exposed to pesticides) were fixed in 5% glutaraldehyde and 2% osmium tetroxide. Then, the ciliates were washed twice with 2.5% glutaraldehyde, fixed in 2.5% glutaraldehyde for 60 min, and washed three times in sodium cacodylate buffer (0.2 mol $L^{-1}$, pH 7.0). The cells were dehydrated through an alcohol series. Samples were cut with the Ultracut Reichert-Jung ultramicrotome (Reichert-Jung, Mannheim, Germany), stained with uranyl acetate and lead citrate, and viewed under a FEI Tecnai $G^2$ transmission electron microscope (FEI, Hong Kong, China).

## 3 Results

### 3.1 Median-Lethal Concentration ($LC_{50}$)

*Urostyla grandis* had different sensitivities to chlorpyrifos and dimethoate, but the mortality rate increased with increasing concentrations of both pesticides (Table 1).

Table 1 Effects of a 24-h acute chlorpyrifos and dimethoate treatments on *Urostyla grandis* ($n$ = 100)

| Pesticide | Concentration (mg $L^{-1}$) | Mortality (%) |
|---|---|---|
| Chlorpyrifos | 0.0212 ± 0.003 | 10.0 |
| | 0.0244 ± 0.004 | 15.0 |
| | 0.0276 ± 0.04 | 38.0 |
| | 0.0308 ± 0.002 | 62.0 |
| | 0.0340 ± 0.003 | 79.0 |
| | 0.0372 ± 0.006 | 94.0 |
| Dimethoate | 0.50 ± 0.030 | 2.0 |
| | 0.55 ± 0.016 | 9.0 |
| | 0.60 ± 0.024 | 4.40 |
| | 0.65 ± 0.071 | 6.20 |
| | 0.70 ± 0.080 | 80.0 |
| | 0.75 ± 0.026 | 96.0 |

The 24-h $LC_{50}$ value of chlorpyrifos, calculated based

Table 2 Median lethal concentrations and fission time effective concentrations of chlorpyrifos and dimethoate to *Urostyla grandis* ($n$ = 100)

| Pesticide | $LC_{50}$ (mg $L^{-1}$) | 95% confidence interval (mg $L^{-1}$) | SC (mg $L^{-1}$) |
|---|---|---|---|
| Chlorpyrifos | 0.029 ± 0.006 | (0.028–0.030) ± 0.0031 | (0.0075–0.0093) ± 0.0006 |
| Dimethoate | 0.685 ± 0.005 | (0.679–0.692) ± 0.0045 | (0.2640–0.2750) ± 0.0003 |

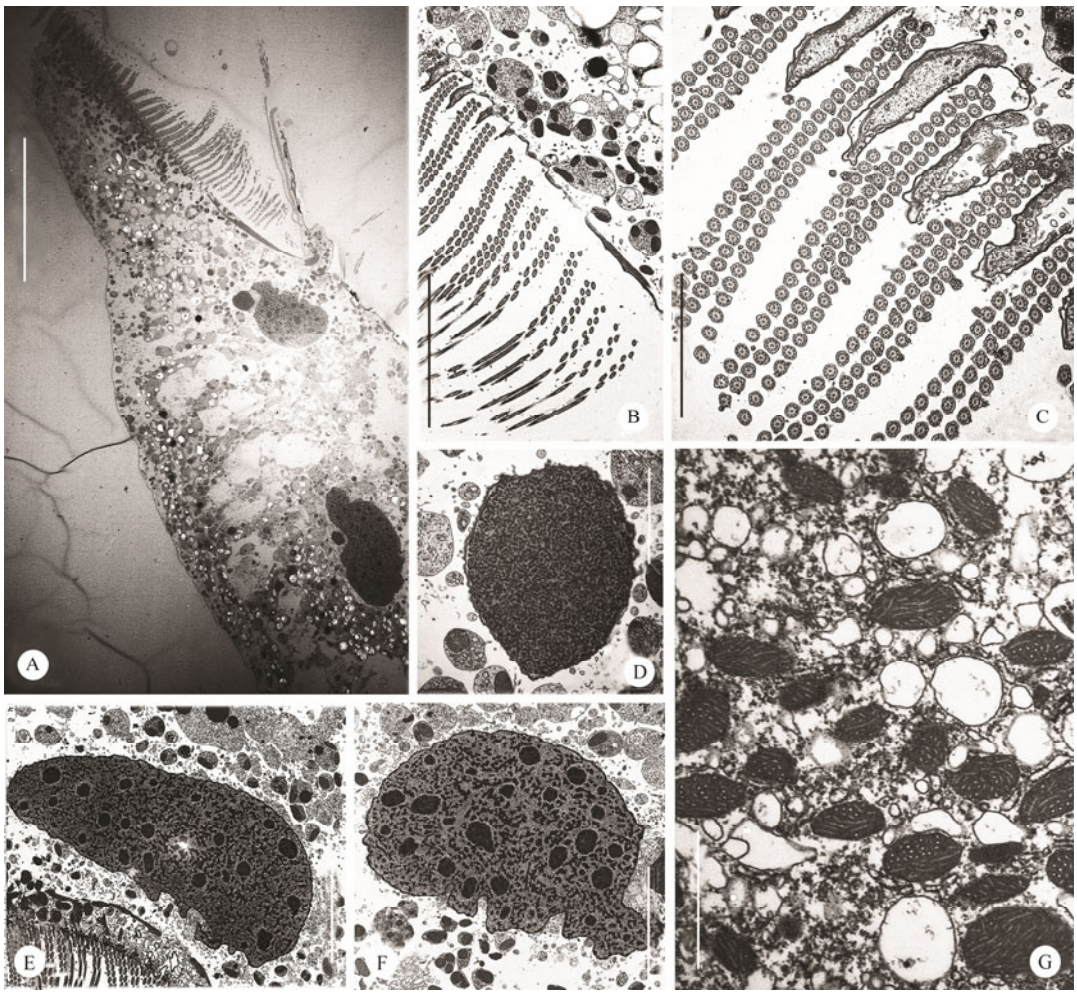


Fig.1 Ultrastructure of *Urostyla grandis* without pesticide treatment. (A) Ventral view of a representative specimen. (B, C) Adoral zone of membranelles. (D) Micronucleus. (E–F) Regular macronucleus. (G) Regular mitochondria. Bars, 30 μm (A), 2 μm (B, C), and 10 μm (D–G).

on logarithmic scale concentrations, was 0.029 mg $L^{-1}$ (Table 2; Fig.1). Almost all ciliates survived after a 24-h exposure to 0 and 0.0212 mg $L^{-1}$ chlorpyrifos, whereas about 20% and 5% of cells survived after a 24-h exposure to 0.0340 and 0.0372 mg $L^{-1}$ chlorpyrifos, respectively.

The 24-h $LC_{50}$ value of dimethoate was 0.685 mg $L^{-1}$ (Table 2; Fig.1). Almost all cells survived after 24-h exposure to 0 and 0.50 mg $L^{-1}$ dimethoate, whereas about 20% and 3% of cells survived after a 24-h exposure to 0.70 and 0.75 mg $L^{-1}$ dimethoate, respectively.

### 3.2 Fission Time Effective Concentration

The fission time effective concentration of chlorpyrifos was 0.0075–0.0093 mg $L^{-1}$ and that of dimethoate was 0.264–0.275 mg $L^{-1}$. Thus, *U. grandis* was more sensitive to dimethoate than to chlorpyrifos. In addition, the fission time effective concentrations of both pesticides were lower than the respective 24-h $LC_{50}$ values (Table 2).

Significant differences were found between the chlorpyrifos treatment group and the control group after the 168-h exposure. Cells exposed to the fission time effective concentration of chlorpyrifos (0.60±0.06) showed significant lower fission time than cells in the control group (1.64±0.05; $P<0.05$; Fig.2). The fission time of cells exposed to the fission time effective concentration of dimethoate was slightly higher (0.74±0.04) than that of ciliates exposed to chlorpyrifos, but was significantly lower than that of the control group (1.64±0.52) ($P<0.05$; Fig.3).

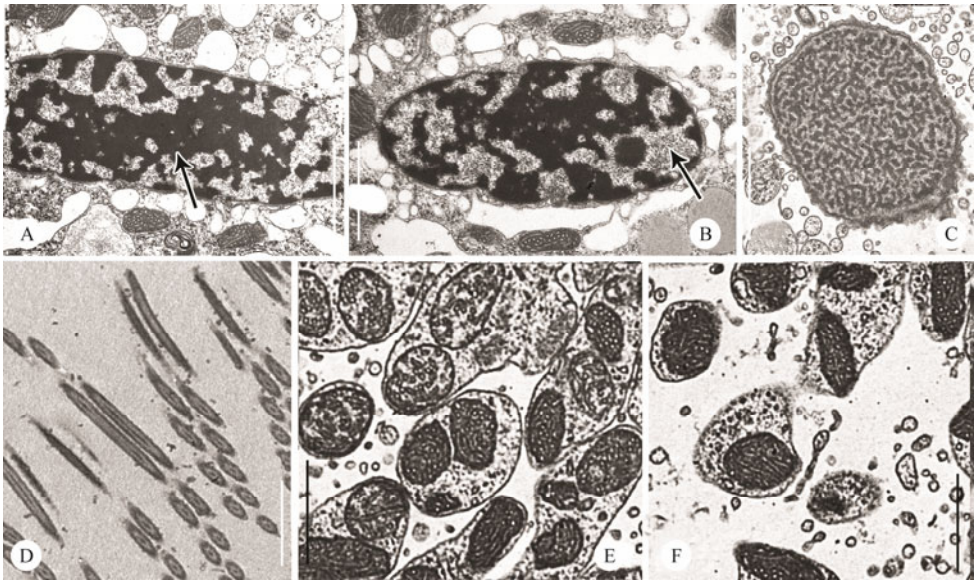


Fig.2 Ultrastructure of *Urostyla grandis* after chlorpyrifos treatment. (A, B) Macronucleus; arrows show condensed chromatin; arrowheads mark sparse chromatin. (C) Micronucleus. (D) Dropped and deformed adoral zone of membranelles. (E, F) Unclear internal mitochondrial structure. Bars, 10 μm (A–C), 5 μm (D), 2 μm (E, F).

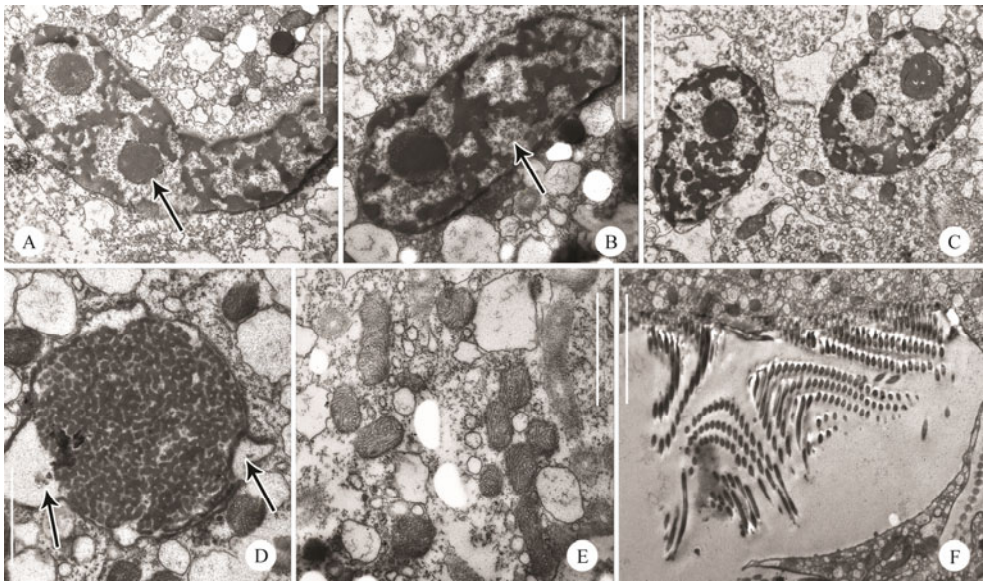


Fig.3 Ultrastructure of *Urostyla grandis* after dimethoate treatment. (A) Macronucleus; arrow shows fusing nucleolus. (B) Macronucleus; arrowhead shows condensed chromatin. (C) Discontinuous macronuclear membrane. (D) Micronucleus; arrows show protruding membrane. (E) Unclear internal mitochondrial structure. (F) Dropped and deformed adoral zone of membranelles. Bars, 10 μm (A, B), 2 μm (D), and 10 μm (E).

### 3.3 Effects of Chlorpyrifos and Dimethoate on *U grandis* Ultrastructure

Cells exposed to chlorpyrifos and dimethoate showed different degrees of ultrastructural abnormities, including the cell membrane system and chromatin anomalies. Cells in the control group had (1) a regular adoral zone of membranelles (Fig.1sa–c); (2) clear and complete macronuclear (Figs.1a, e, and f) and micronuclear (Figs.1a and d) membranes; (3) homogeneous chromatin in the macronucleus and micronucleus (Figs.1d, e, and f); and (4) regular mitochondria (Fig.1g). In the cells treated with chlorpyrifos, the nuclear membranes were disrupted; the chromatin condensed, the macronucleus was separated from the nucleoli, the internal structure of the mitochondria lost its integrity, and the adoral zone of membranelles became deformed (Fig.2). Cells treated with dimethoate showed similar abnormalities as those treated with chlorpyrifos (Fig.3), such as the membranes of the micronucleus also protruded (Fig.3d), the membranes of the mitochondria were indistinct (Fig.3e), and the adoral zone of membranelles deformed (Fig.3f).

## 4 Discussion

Chlorpyrifos and dimethoate are broadly used to control aphids, leafhoppers, and whiteflies (Singh *et al.*, 2004; He *et al.*, 2013). As these products are relatively massively used in the market, many toxicological cases have been reported (Ki *et al.*, 2013; Zhuang *et al.*, 2015; Chen *et al.*, 2016). However, their direct or indirect effects on aquatic organisms are not fully studied. Ciliates have long been used as water quality indicator (Lynn and Gilron, 1992). The $LC_{50}$ value is a commonly used measure for toxicity of potential harmful substances, particularly in fish, amphibians, and zooplankton (Fogels and Sprague, 1977; Huertas *et al*., 2002; Kir *et al*., 2004; Ugine *et al*., 2005). However, this method can be time-consuming, labor-intensive, and costly (Madoni, 2000). Consequently, a series of alternative methods have been developed that are more rapid and efficient (Erkin *et al*., 2012; Shi *et al*., 2013; Chatterjee *et al*., 2016; Han *et al*., 2016; Martínez-Domínguez *et al*., 2016). Chlorpyrifos and dimethoate are widely used insecticides; thus, it is necessary to study their potency and sensitivity on target animals as well as the suitable method of using toxicological data in a single cell eukaryote. In the present study, a bioassay protocol was developed to assess the effectiveness of chlorpyrifos and dimethoate using ciliates as the target animal, and the fission time effective concentration was determined.

In our results, the fission time effective concentrations of chlorpyrifos and dimethoate to *U. grandis* were clearly lower than the $LC_{50}$ values in the laboratory study. The fission time effective concentration might be more suitable than $LC_{50}$ for water quality monitoring. Therefore, the results and comparisons indicate that the safe concentration was lower than the $LC_{50}$ value.

In addition, our results suggest that chlorpyrifos and dimethoate affect cell division of *U. grandis*, but may not lead to death, so there is some tolerance of ciliates to organophosphorus pesticides. Furthermore, the present study illustrates that ciliates can be used as organisms for biological monitoring. Many studies have shown that ciliates are tolerant to harmful substances in the environment. For example, *Pseudocohnilembus persalinus* can resist to oxytetracycline (OTC) and *Tetrahymena thermophila* can also resist to the oxidative stress (Huang *et al.*, 2016; Wang *et al.*, 2017). Some quantitative and morphological differences can be identified between the cells in the control group and those in the experimental groups when they are treated with different fission time effective concentrations. Our experiment provides a new method for toxicological monitoring of single-celled organisms; that is, the ciliate fission time is affected by toxic stress, and this effect is an important manifestation of cell toxicity.

Several studies have reported the effects of chlorpyrifos on aquatic organisms at the cellular level. Chlorpyrifos causes damage to the cell walls, leading to the lipid accumulation, the formation of electrodense bodies, and an increase in the size and number of starch granules in algae (Asselborn *et al*., 2015). Ciliates, as unicellular microorganisms, are characterized by nuclear dimorphism, possessing one or more micronuclei and macronuclei (Morgens *et al*., 2014). During the vegetative growth, the macronucleus is responsible for all nuclear transcription. Damage to the macronucleus has been reported by Hussain *et al*. (2008) in cells of *Paramecium caudatum* exposed to carbofuran (Hussain *et al*., 2008). Macronuclei can be restructured, fragmented, scrambled, rejoined, degraded, rebuilt, and amplified in various ways and are controlled epigenetically (Yao *et al*., 2003; Nowacki *et al*., 2008; Swart *et al*., 2013). Our study show that macronuclear and macronuclear membranes are damaged in ciliates after pesticide exposure. These results confirm that disrupted membranes are a warning signal for the effects of toxicants on the aquatic systems because they have a sensitive reaction to environmental stresses (Sabater *et al*., 2007). The different phenomena between the macronuclear membrane in ciliates exposed to the chlorpyrifos and dimethoate indicate that the ciliates have distinct reactions to various pesticides. The chromatin condensation shown in our study is a characteristic of apoptosis in protozoans, which is easily observed under a microscope (Al-Olayan *et al*., 2002).

The mitochondria appeared differently after the chlorpyrifos and dimethoate treatments: the mitochondrial matrix gathered to form one to several globes in chlorpyrifos-treated cells, whereas the mitochondrial outer membrane developed more wrinkles in dimethoate-treated cells. This difference may be due to the different degenerative effects caused by the two treatments. Ciliates treated with chlorpyrifos and dimethoate exhibited an unclear internal structure and a degenerative mitochondrial pattern. Our results are consistent with those of Martín-González *et al*. (2005), who found that ciliates developed a mitochondrial degenerative pattern after Cd and Zn exposure. Studies on rats have shown that high doses of CPF can lead to abnormal mitochondria, such as

swelling, fragmentation, and diminution (Xu *et al.*, 2017). Both the chlorpyrifos and dimethoate treatments resulted in a dropped and deformed adoral zone of the membranelles. Thus, the effects of organophosphates on cell structure and organelles are not only restricted to the target organisms, they also extend to non-target organisms, which play a pivotal role in the ecosystem (Asselborn *et al*., 2015).

## 5 Conclusions

We measured the $LC_{50}$ values following a 24-h exposure to different concentrations of pesticides including chlorpyrifos and dimethoate in the ciliate *U. grandis* under laboratory conditions. Subsequently, the fission time effective concentrations of chlorpyrifos and dimethoate during cell division were investigated after 24-, 48-, 96-, 120-, 144- and 168-h exposures using fission time measurements. We conclude that the fission time effective concentration test is a rapid and novel method to evaluate the effects of environmental pollutants, based on a comparison with derived $LC_{50}$ values (24 h). Additionally, this is the first study to use an ultrastructural analysis to observe the transformation of cellular system in ciliates exposed to chlorpyrifos and dimethoate. These results also indicate the potential use of ciliates as a biomonitor to survey pesticide pollution in agriculture in the future.

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