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Toxic effects of chemical pesticides (trichlorfon and dimehypo) on *Dunaliella salina*

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A B S T R A C T

Dunaliella salina, a unicellular green alga of environmental tolerance, was employed as test organism to investigate the toxicity effects of trichlorfon and dimehypo widely used in agriculture and veterinary as pesticides. The influences of trichlorfon and dimehypo on cell growth, β-carotene level, cell morphology changes, and activities of superoxide dismutase (Sod) and catalase (Cat) were investigated. At the concentrations less than 0.050 g L^{-1} trichlorfon or 0.0005 g L^{-1} dimehypo, cell responses were similar to control. When treated with 0.075–0.100 g L^{-1} trichlorfon or 0.001–0.004 g L^{-1} dimehypo, cell growth and β-carotene levels declined at first and then revived. When concentrations were higher than 0.125 g L^{-1} trichlorfon or 0.005 g L^{-1} dimehypo, both cell growth and β-carotene levels decreased until they were undetectable. The 10-d IC50 of trichlorfon and dimehypo on *D. salina* were 0.179 g L^{-1} and 0.032 g L^{-1}. Both pollutants could stimulate the increase of Cat activity at a low concentration. Tolerance of *D. salina* to trichlorfon was obviously higher than that of dimehypo.

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1. Introduction

Trichlorfon [dimethyl-(2,2,2-trichloro-1-hydroxyethyl) phosphonate] is a selective organophosphate insecticide used to control a variety of arthropod pests including cockroaches, crickets, silverfish, bedbugs, fleas, cattle grubs, flies, ticks, leaf miners, and leaf-hoppers. It is available in granular, soluble concentrate and wettable powder formulations and is used in agriculture and veterinary, that acts onto arthropods as an inhibitor of acetylcholinesterase (E.C. 3.1.1.7) (Lopes et al., 2006). Dimehypo (disodium 2-methylaminotrimethylene dithiosulfonate), an organonitrogen insecticide for use on rice and other crops, has a similar structure to nereistoxin and, in China, is replacing benzene hexachloride (BHC) (Tang, 1980, 1982). Chemicals released into the environment from agriculture and aquaculture activities are responsible for adverse ecological effects if the concentrations are higher than a threshold of environmental self-purification and organism tolerance. Pesticides represent a threat to living organisms including algae and its biochemical systems, since these compounds are able to be bioconcentrated by the aquatic biota (Jonsson and Toledo, 1993; Jonsson et al., 2001). Thus, it is of significance to find effective methods for testing and monitoring agricultural chemical pollutions in aquatic environments.

Algae are important components of the primary production and detrimental effects in these organisms may affect the entire food chain (Jonsson and Aoyama, 2007). Even a very low concentration of organic pollutants in the water can exert detrimental influences on algae. It could be elucidated as follows: cell membrane damaged by the toxicants leads to augmented osmosis, and then various surrounding toxic substances enter into the internal of the cell and inevitably react with some vital active compositions. In addition, they could also bring about an abundance of active oxygen inside the cell and over-oxidize the membrane lipid (Zhu and Jiang, 2009). Nowadays, the representatives for the algae are mainly used microalgae (ISO, 1995).

Dunaliella salina, one member of the genus *Dunaliella* (Chlorophyceae, Volvocales), is an extremely halotolerant, unicellular, green and motile algae. The genus *Dunaliella* possesses unique and remarkable ability to survive in extreme conditions such as a wide range of NaCl concentrations ranging from about 0.05 M to saturation (around 5.5 M), intense light, high temperature and acid–base environments with broad tolerance scope of pH values from 1 to 11 (Avron, 1992; Fraser and Bramley, 2004; Zhu and Jiang, 2009). Another remarkable characteristic of *D. salina* is that it can excessively accumulate large amounts of β-carotene in cells under extreme osmotic stress to maintain its osmotic balance, which makes it one of the best sources of natural β-carotene (Ye et al., 2008). Also, the synthesis or elimination of another osmolyte glycerol to an intracellular concentration osmotically balancing the external salinity permits the cells to resume growth (Avron, 1992). These characteristics give *D. salina* an obvious applicable value as test organism in the research field of environmental toxicology.

The aim of this study was to test the effects of two significant agricultural pesticides (trichlorfon and dimehypo) on the environmentally tolerant *D. salina*. Dose–effects of the two pesticides on the survival of *D. salina* and its contents of β-carotene, activities of Sod and Cat were observed to evaluate the environmental effects

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of both pesticides. It is hoped that the results of present research would provide useful references for the usage and environmental control of both pesticides.

2. Materials and methods

2.1. Algal culture

D. salina strain 435 (UTEX 200) was obtained from Institute of Hydrobiology, Chinese Academy of Sciences. Cells of *D. salina* were cultivated in the defined medium (Sheffer and Avron, 1986) containing 1.5 M NaCl, which was sterilized at 121 °C for 30 min before inoculation, at 26 °C and 8000 lx provided by cool-white fluorescent lamps, under a 14/10 h light/dark cycle with shaking at 96 rpm.

2.2. Toxicity assays

Cells of *D. salina* were collected at the log phase or late log phase. An equal volume of fresh culture medium of 50 mL in the presence of each pollutant at various concentrations was added to the algal pellets in 150 mL of previously sterilized conical flask. The corresponding trichlorfon concentrations were 0.025, 0.050, 0.075, 0.100 and 0.125 g L^{-1}, and the dimehypo concentrations were 0.0005, 0.001, 0.002, 0.004 and 0.005 g L^{-1}, respectively. The algal culture without any pollutant was used as control. All cultures were cultivated followed the culture condition described above. The cells were observed microscopically.

The absorbance of *D. salina* cultures with concentration gradient were measured at 630 nm, and 2 mL of each culture with concentration gradient were filtrated by 0.45 μm filter membranes, which were weighed and dried. The filter membranes were rinsed by deionized water and then dried at 80 °C for 12 h. The dried weight of cells in 2 mL cultures with concentration gradient were obtain by weighing dried filter membranes and subtracting the weight of filter membranes without algal pellets. The relationship curve of the dried weight of cells (g L^{-1}) (y) against OD_{630} value (x) was plotted and the biomass was calculated according to the regression by the following equation:

$$y = 0.6327x + 0.368, \quad R^2 = 0.9843. \tag{1}$$

The cell numbers of cultures with concentration gradient were calculated by blood cell count. The relationship curve between OD_{630} and cell number was plotted, and the cell number was obtained by regression by the following equation:

$$Y = 3418.3X + 226.33, \quad R^2 = 0.9908, \tag{2}$$

where Y represents cell number ($\times 10^4$) and X represents OD_{630} value.

The cell number and biomass of both samples and control were measured every day (per 24 h). Both cell numbers and biomass were determined by optical density method. The absorbance of samples and control at 630 nm were measured every day, and cell number and biomass were calculated from the relationship curve between the dry weight of cells and OD_{630} and the relationship curve between OD_{630} and cell number.

2.3. β-carotene analysis

The standard curve for β-carotene was obtained with different OD_{453} values against the corresponding concentrations of the β-carotene/acetone standard solutions. The regression Eq. (3) was

$$y = 3.5224x - 0.0349, \quad R^2 = 0.9976, \tag{3}$$

where y is β-carotene content (mg L^{-1}) and x is OD_{453} value.

Extraction of β-carotene from *D. salina* culture followed previously described protocols (Ben-Amotz and Avron, 1989; Cowan and Rose, 1991). A cell pellet of 2 mL of algal culture harvested by centrifugation at 6000 g for 10 min was dissolved in acetone, followed by agitating until all pigments were extracted. Saponification of the pigment mixtures was performed in 60% (w/v) ethanol/KOH (9:1) for 3 h at 4 °C. The β-carotene was extracted with ether after the addition of 1.2% NaCl, dried, and dissolved in acetone to measure its OD_{453} value, which was then converted into β-carotene concentration based upon the regression equation.

2.4. Enzyme extraction

The crude extracts were perpetrated according to the methods of Lee and Bennett (1982) with some modifications. Cells of *D. salina* treated with pollutants in cultures after 2 d (48 h) were harvested by centrifugation at 6000*g* for 10 min at room temperature. Half a gram of algal pellets were transferred to a 2 mL centrifuge tube, and then resuspended with 1 mL sodium phosphate buffer (50 mM, pH 7.8) and 0.02 g polyvinylpyrrolidone (PVP). The suspension was centrifuged at 12 000*g* for 20 min at 4 °C to crumble cells, and the supernatant was collected by centrifugation at 13 000*g* for 30 min at 4 °C. The supernatant was enzyme extract and used for enzyme activity analysis directly or diluted with glycerol by 50% (w/v) to maintain its activity at −20 °C until the operation of activity analysis.

2.5. Sod activity analysis

Sod activity was analyzed according to the methods of Constantine and Stanley (1977) and Hao (2006) with some modifications. The reaction mixture contained 1 mL of methionine (39 mM), 15 μL of enzyme extract, 1 mL of nitroblue tetrazolium (NBT, 189 μM) and 1 mL of riboflavin (6 μM). The mixtures were illuminated by fluorescent lamp (4000 L×) for 20 min and then the absorbance was determined at 560 nm. The blank contained 1 mL of methionine (39 mM), 1 mL of NBT (189 μM) and 1 mL of sodium phosphate buffer (50 mM, pH 7.8). The solution which contained 1 mL of methionine (39 mM), 15 μL of sodium phosphate buffer (50 mM, pH 7.8), 1 mL of NBT (189 μM) and 1 mL riboflavin (6 μM) served as control. The solution which contained 1 mL of methionine (39 mM), 15 μL of enzyme extract, 1 mL of NBT (189 μM) and 1 mL of sodium phosphate buffer (50 mM, pH 7.8) was used as pigment value. One unit (U) of Sod was defined as the amount of enzyme that caused a 50% decrease of the Sod-inhibitable NBT reduction.

Sod activity is calculated as the by the following equation:

$$\text{Sod activity}(\text{U g}^{-1}) = \frac{(A_C - A_S + A_P) \times V}{\frac{1}{2} \times A_C \times FW \times V_t} \tag{4}$$

where A_C is the absorbance of control, A_S is the absorbance of sample, A_P is the absorbance of pigment value, V is total enzyme extract volume (mL), V_t is enzyme extract volume in sample reaction (mL), and FW is sample fresh weight (g).

2.6. Cat activity analysis

Cat activity was analyzed according to the method of Hao (2006) with some modifications. Cat activity was assayed at 25 °C in a reaction mixture containing 1 mL of enzyme extract and 2 mL of 1 mM H_2O_2. The solution which contained 1 mL of enzyme extract and 2 mL of sodium phosphate buffer (50 mM, pH 7.8) was used as blank. Activity was determined by UV spectrophotometer at 240 nm, measuring the decrease in absorbance every

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