EFFECT OF DECHLORANE PLUS ON PSEUDOKIRCHNERIELLA SUBCAPITATA BY FLOW CYTOMETRY

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Introduction

Dechlorane Plus (DP) was developed as an additive chlorinated flame retardant to substitute for Mirex which was banned in the 1970s[1]. It has been used in coatings for commercial electrical wires and cables, connectors for computers, and plastic roofing material for at least 40 years[2] The worldwide annual production volume of DP is estimated to be 10 million pounds and categorized as a high-production-volume (HPV) chemical by the U.S. Environmental Protection Agency[3].The increase in DP use is expected because it was recently included in a European Commission report as a potential candidate to replace the now restricted decabromodiphenyl ether flame retardant[2]. Despite its commercial longevity, DP was first identified in the environment until 2006[4]. From then, more and more studies indicated that DP is a ubiquitous pollutant which has been found in various environmental matrices such as air, water, sediment, dust, aquatic organisms and even in human hair, serum and milk. However, toxicity data for DP are scarce until now and none of the studies assessed toxicity in an algae species.

Flow cytometry (FCM) is a rapid method for the quantitative measurement of light scattering and fluorescent properties of cells. One of the major applications of flow cytometry to environmental studies has been in the fields of ecotoxicity using microalgae, where it has been used to assess the effects of toxicants on algal cells based on their different photosynthetic pigments and light-scattering properties[5]. It can perform multi-parameter analysis on a wide range of cell properties by measuring algal cell densities, light scatter signals and chlorophyll a autofluorescence, after excitation at 488 nm. Through the use of biochemically specific fluorescent dyes, flow cytometry can also provide information about the physiological status of cells and the mechanisms of action of toxicants. For example, algal esterase activity can be determined by detecting cell fluorescence from 530–560 nm, after staining cells with fluorescein diacetate (FDA). H_2DCFDA is the membrane-permeable probe which produces a green fluorescent dye in the cell after the acetate groups have been removed by intracellular esterase and oxidation has occurred by reactive oxygen species (ROS). Therefore, the changes of cell fluorescence can be used to assess the amount of intracellular ROS. Propidium iodide (PI) was utilized to assess cell membrane integrity. In this paper, we investigated the effects of DP on freshwater algae *Pseudokirchneriella subcapitata* by FCM to assess its aquatic ecological risk.

Materials and methods

The strain, *P. subcapitata* were cultured in the Institute of Environmental Systems Biology, Dalian Maritime University. All stock cultures were grown in 250-mL Erlenmeyer flasks on a 12:12 h light/dark cycle at 3000Lux at 23±1℃. Cultures were used till microorganisms were in exponential growth phase of growth. The cell densities in all three algal samples were adjusted to approximately 10^5cells/mL for FCM analysis.

Dechlorane Plus was provided from Jiangsu Anpon Electrochemical Co., LTD. Due to the lower water solubility of DP, vehicle for DP is necessary in biological assays. Prior to study initiation, test batches of DP were prepared using multiple vehicles to determine the most appropriate for algal exposure. Based on this assessment, <0.1% acetone (v / v) was used as the vehicle control in this study. DP stock solution was prepared in acetone and its concentration was determined by GC/MS. Serial dilutions were prepared by algal culture medium at nominal concentrations of 13.51, 135.1 and1351 ng/L.

After 2, 24, 48 and 72 hours (h) exposure, effects of DP on cell membrane integrity, esterase activity, intracellular ROS generation and chlorophyll a fluorescence of *P. subcapitata* were investigated. The method of fluorescence dye was according to Xiao et al[6]. The PI stock solution (50 mg/L) was prepared by dissolving PI (Sigma, Cat No. F4170–10MG) into Milli-Q water and stored at 4℃ until use. PI stock solution was added into 1 ml of algal samplings to give final concentrations of 10 μ mol/L. The FDA stock solution (10 g/L) was prepared by dissolving FDA (Sigma, Cat No. F7378-5G) in a reagent grade acetone with the aid of a vortex mixer and the concentration of working solution is 25 μ mol/L for 8min incubation at 25℃. The H_2DCFDA was dissolved in Dimethyl sulfoxide (DMSO) to achieve the final concentration as 100 μ mol/L after 1h incubation at 37℃.

The fluorescein intensity was measured using flow cytometry (BD-FACSCalibur, Becton Dickinson BioSciences, San Jose, and CS, USA). Cells were excited with an argon excitation laser (488 nm). The fluorescein converted from FDA and H_2DCFDA was detected in the FL1 detector (515–545 nm). Fluorescence of PI was detected in the FL2 detector (564–606 nm), and the chlorophyll a fluorescence was detected in the FL3 detector (>650 nm). The flow cytometer was operated using a constant flow rate of 12 μ L/min, and at least 10000 events were tested for every sample. Data were expressed as mean fluorescence intensity per cell (MFI). All experiments were performed in three replicates. Data were presented in means ± SD. Because there was no prominent difference between blank and vehicle control, the vehicle control was used here to instead of blank control. Significant differences between vehicle control and treated samples were determined by ANOVA. Differences were considered to be significant at $p < 0.05$.

Results and discussion

Cell membrane integrity

PI is a nuclear stain which embedded in double-stranded DNA to release red fluorescence. It can not pass the intact cell membrane therefore has been used to determine algal cell viability and cell membrane integrity. The relative fluorescence intensity of PI in algal cells was showed in Fig. 1. After 2h of DP exposure, the changes of fluorescence intensity appeared dose-dependant manner and was increased significantly in highest DP concentration which was indicated the damage of algal cell membrane. But in following exposure times, there was no significant change at any doses. It seemed that the damage in cell membrane was minor and recovered. And the cell membrane integrity implied there was no fluorescent substance leakage to guarantee the accuracy of following assays.

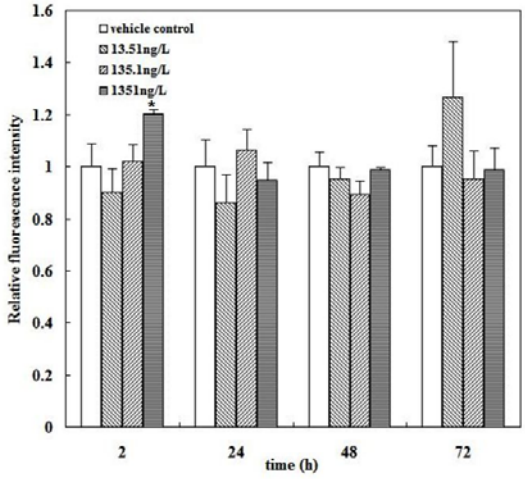

Fig. 1. Effects on relative fluorescence intensity of PI at different DP concentrations. Error bars are the standard deviation (n=3). An asterisk (*) indicates a significant difference among treatments ($p < 0.05$).

Esterase activity

Esterase activity was estimated as the mean rate of conversion of FDA to fluorescein and was expressed as the rate achieved by the algal cells with DP treatment relative to that achieved by the vehicle control cells. The relative fluorescence intensity was increased at higher concentrations groups after 2h and 24h exposure (Fig. 2) which indicated esterase activity was induced by DP treatment. But at 48 and 72h treatments, the esterase activity did not increase significantly. No obvious dose-effect appeared. Esterase activity is a sensitive indicator to imply the effect of toxicants. In this study, the results showed DP induced increase in enzyme activity in early exposure time but did not impaired it with increasing exposure intensity.

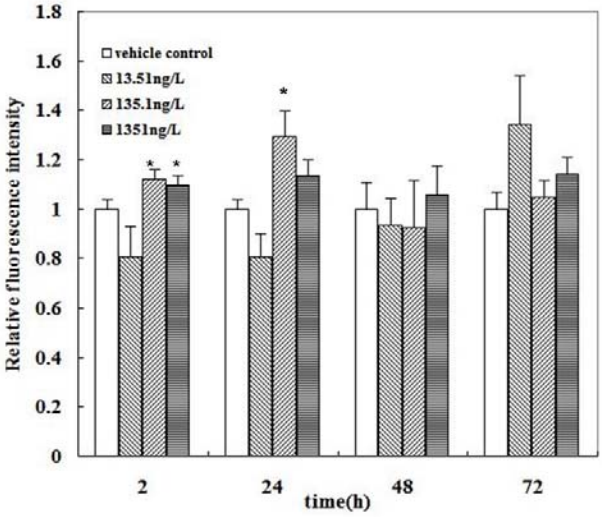

Fig. 2. Effects on esterase activity at different DP concentrations. Error bars are the standard deviation (n=3). An asterisk (*) indicates a significant difference among treatments ($p < 0.05$).

Intracellular ROS generation

The relative fluorescence intensity of DCF, an indication of intracellular ROS which indicated the oxidative stress in algal cells was showed in Fig. 3. At 48h of DP exposure, the intracellular ROS increased significantly at 13.51 and 135.1ng/L groups, whereas decreased dramatically at 1351ng/L group. The intracellular ROS was balanced by the antioxidant defenses in cells. It was supposed that the accumulation of intracellular ROS induced the antioxidant defenses scavenging free radical. Therefore, ROS generation was decreased significantly in highest concentration DP group.

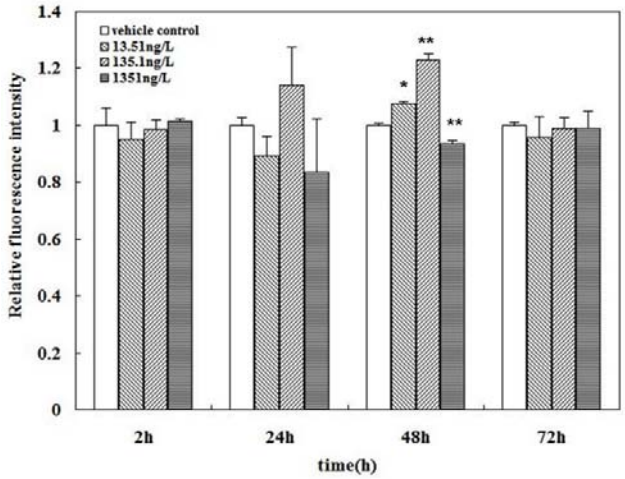

Fig. 3. Effects on intracellular ROS generation at different DP concentrations. Error bars are the standard deviation (n=3). An asterisk (*) indicates a significant difference among treatments ($p < 0.05$), and two asterisks (**) indicates a extremely significant difference among treatments ($p < 0.01$).

Average chlorophyll a content

The changes of average chlorophyll a content was showed in Fig. 4. At 2h exposure, DP treated algal cells decreased the chlorophyll a content although it was not significant. But it was recovered in the following exposure times. At highest DP concentration group, the average chlorophyll a content increased significantly and showed the so-called hormesis.

Combined to the above results, it was concluded that DP is a low-toxic compounds and has marginal effect on *P. subcapitata.*

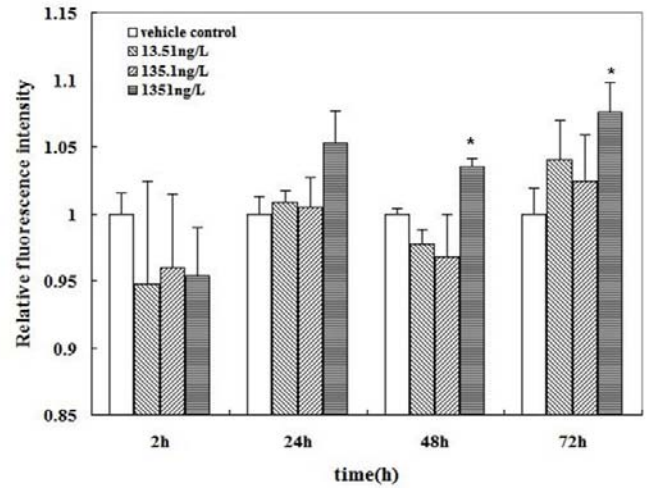

Fig. 4. Effects on average chlorophyll a content at different DP concentrations. Error bars are the standard deviation (n=3). An asterisk (*) indicates a significant difference among treatments ($p < 0.05$).

Acknowledgements

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