Oxidative stress and growth behavior responses of marine diatoms Phaeodactylum tricornutum and Skeletonema costatum to three typical persistent organic pollutants

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Abstract

To study the oxidative stress responses and growth behavior of marine diatom to persistent organic pollutants (POPs), *Phaeodactylum tricornutum* and *Skeletonema costatum*, two species of algae which are potentially harmful to marine environment as red tide algae, were chosen as test diatoms against three common kinds of POPs in the ocean: Aroclor 1254, benzo[a]pyrene (BaP), and dichlorodiphenyltrichloroethane (DDT). The two algae were cultivated in three concentrations of 50, 100 and 500 μg L^{-1} for 72 h; and the temporal production of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and peroxidase (POD) were determined. Results showed that MDA responded quicker than SOD in *P. tricornutum* and *S. costatum*, a peak of MDA was detected after 2 h of exposure, while the activity of SOD peaked after 12 h of exposure. For studying the growth behavior of diatoms in the bi-algal culture to POPs, two species in different initial cell densities were investigated in 28-day exposure to the three POPs in concentrations of 15 and 50 μg L^{-1}. The growth of *P. tricornutum* increased significantly only in the medium enriched with 50 μg L^{-1} DDT ($ratio_{max}$ = 148%). On contrast, the cell density of *S. costatum* increased during the most time of exposure regardless of initial cell density. The results demonstrate that POPs toxicity could cause oxidative stress even cell damage in both *P. tricornutum* and *S. costatum*, but algal growth was promoted by the POPs in the mixed culture, which indicated that the presence of pollutants in the sea was an important inducement of the species groups change. The results of this study may provide valuable help for detailed studies of oxidative stress and growth behavior responses of marine diatom to POPs.

Keywords-Oxidative stress; Aroclor 1254; Benzo[a]pyrene; Dichlorodiphenyltrichloroethane; Marine diatom

I. INTRODUCTION

P. tricornutum and *S. costatum*, eukaryotic marine diatoms, are potentially harmful to marine environment as red tide algae [1-3]. Many physiological and ecological studies on the algae in pollutants exposure have been conducted using these two species[4]. *P. tricornutum* is an important food resource for marine zooplankton, and is also used as a stand test material in toxicological studies[5]. Furthermore, *P. tricornutum* is the second diatom species whose genome was completely sequenced[6], which facilitated its use in the field of ecotoxicology. *S. costatum* is also often used in toxicity tests due to its presence in oceans worldwide.

In this study the three POPs (Aroclor 1254, BaP and DDT) were chosen as model substances due to their wide presence as environmental contaminants[7-10]. Previous studies showed that POPs harmed animals in the following ways. Aroclor1254 affects the auditory system[11], BaP affects both mitochondrial and nuclear DNA, and DDT and its metabolites to induce apoptosis in different cellular types[12]. POPs can enter the aquatic ecosystem through effluent, atmospheric deposition, runoff, and groundwater, they have become ubiquitous in the biosphere and have seriously threatened the health of aquatic animals, and consequently had caught the worldwide attention in the recent years[13].

It has been reported that pollution factors in the marine environment accumulated in lipids and induced oxidative stress, which is known as excess of reactive oxygen species (ROS) and lipid peroxidation in algae[14-15]. ROS are produced in different cellular compartments, and are responsible for cell damage in oxidative stress condition, but are rapidly buffered by the antioxidant system. Especially, by antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and antioxidant compounds promoting detoxification[6,11,13]. Thus, SOD and POD activities are measured as indicators in the intracellular level of antioxidant responses. Malondialdehyde (MDA) is a secondary product of lipid peroxidation, which can be induced by ROS, and it has been demonstrated to be a powerful tool for the identification of oxidative reaction[14-15].

The damage of POPs to microalgae at the molecular, cellular, and population levels has been demonstrated [16-19]. In contrast, there are few convincing data with respect to the effects of POPs increasing at the community level and considerable uncertainty still exists. In this article, we conducted bi-algal culture experiments under POPs conditions using several combinations of initial cell densities of the two species, aiming to find out which species is able to dominate the phytoplankton community. This will improve our understanding of the inhibitory and stimulatory interactions between species under pollution conditions.

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Diatoms were separated into several aliquots, each exposed to the three POPs as a dosage to determine dose-response curves for growth inhibition, the other aliquots were exposed to different concentrations of POPs to test the response of the antioxidant system, which relates to the antioxidant level caused by pollutants. Thereafter the levels of MDA and the activities of SOD and POD were determined. Finally, we mixed the cultures of two diatoms and exposed them to various concentrations of the POPs used in this study. We then observed the changes of algae growth behavior in bi-culture under POPs exposure.

II. MATERIALS AND METHODS

Microalgal culture

P. tricornutum and *S. costatum* were obtained from the Institute of Oceanology, Chinese Academy of Sciences. The diatoms were cultured in f/2 medium at 20 ± 0.5°C on a 12/12 h light/dark cycle at 56 μEin/m^2/sec. To increase the cell density, the algae cultures were subcultured weekly to achieve large volume flasks. For experimental purposes, exponentially growing cells were mildly subdivided between each 500 mL Erlenmeyer flasks.

BChemicals preparation

Aroclor 1254 (>99.9% purity), BaP (>99.9% purity), DDT (>99.9% purity) and dimethyl sulfoxide (DMSO, >99.9% purity) used in this study were purchased from Sigma, USA. Stock solution of POPs was prepared using DMSO, which was further diluted to working solutions of different designated nominal concentrations before the exposing experiment.

Experimental design

Microalgae were cultured in f/2 medium. The initial cell density in each flask was about 5×10^5 cells per milliliter. Detection of MDA concentration, SOD activity and POD activity were conducted in triplicate. The data presented here were the average values of three parallel samples and their relative standard deviations were less than 5%. Controls without exposure to POPs were also included because DMSO was investigated previously to have no effect on the growth of *P. tricornutum* and *S. costatum* to reach the highest tested concentration of 0.5%. Algae cells were counted using a hemacytometer under a microscope.

Detection of antioxidant enzyme activities

50 mL algae samples (n=3) were collected after 0, 2, 6, 12, 24, 48 and 72 h of POPs exposure. Algae cells were collected by centrifugation at 3000 *g* for 15 min and then broken down in the phosphate buffer (pH 7.0) by sonication, operation program was described as: work for 3 s, pause for 3 s, and 50 rounds in total. Samples were observed under a microscope to make sure the breaking ratio was more than 95%. Finally, the sonicated solution was centrifuged again at 4000 *g* for 15 min at 4°C and the supernatants were collected to measure the antioxidant enzyme activities. SOD activity was detected using SOD Kit (Jiancheng Bioengineering Institute, Nanjing, China). One unit (U) of SOD (per mg protein) was defined as the amount causing 50% inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The activity of peroxidase was estimated by guaiacol colorimetric method.

Detection of MDA content

The MDA content of algae cells was measured using the thiobarbituric acid (TBA) reactive substances test according to the method described previously [20]. Algae cells were collected by centrifugation at 4000 *g* for 15 min and the precipitation was treated with 0.5% (w/v) TBA at 100°C for 15 min. The mixture was cooled immediately cooled in an ice water bath and again centrifuged again at 4000 *g* for 10 min. The absorbance of the supernatant at 532 nm was recorded and corrected for unspecific turbidity by subtracting the value at 600 nm.

Bi-algal culture experiments

Initial cell densities of the two species and inhibition concentration of three POPs in bi-algal cultures were shown in Table 1. Mixed cultures of *P. tricornutum* and *S. costatum* were dealt with 15 and 50 μg/L of Aroclor 1254, BaP and DDT for 28 days, respectively. Algae cells of each experimental unit were counted at 9:00 pm every day.

TABLE I. THE COMBINATIONS OF INITIAL CELL DENSITIES FOR THE TWO SPECIES AND INHIBITION CONCENTRATION OF THREE POPS

Initial cell density ($\times10^5$ cells mL^{-1})		**Inhibition concentration (μg/L)**		
P. tricornutum	***S. costatum***	***Aroclor 1254***	***BaP***	***DDT***
1±0.2	4±0.3	15, 50	0	0
1±0.3	4±0.1	0	15, 50	0
1±0.1	4±0.3	0	0	15, 50
4±0.2	1±0.1	15, 50	0	0
4±0.3	1±0.1	0	15, 50	0
4±0.1	1±0.1	0	0	15, 50
2.5±0.2	2.5±0.1	15, 50	0	0
2.5±0.3	2.5±0.2	0	15, 50	0
2.5±0.1	2.5±0.1	0	0	15, 50

One group of *P. tricornutum* and *S. costatum* mixed culture was dealt with 15 and 50 μg/L of Aroclor 1254, BaP and DDT, respectively.

Statistical analyses

Data were expressed as means and standard deviation of three replicates. The data between different treated groups in each measurement were compared statistically by one-way analysis of variance (ANOVA). The statistical analyses were performed with SPSS 13.0.

III. RESULTS

Detection of antioxidant enzyme activities

The activities of SOD increased in both *P. tricornutum* and *S. costatum* when exposed to the three POPs, Aroclor 1254, BaP and DDT, respectively (Figs.1, 2). It is important to highlight that these increments were detected after 12 h of exposure, with maximum levels 2.59-3.74 times higher than in the control in *P. tricornutum* (Fig.1). The increased level depended upon different pollutants and different concentrations of the POPs in the medium. However, the highest activity of SOD did not always occur at the highest concentration of POPs exposure. SOD activity peaked when Aroclor 1254

concentration was 100 μg L^{-1} in medium, 3.58 times of the control, while the SOD level decreased with the continued increase of Aroclor 1254 concentrations (2635 U mg^{-1} Protein in 100 μg L^{-1}, 2976 U mg^{-1} Protein in 500 μg L^{-1}) (Fig.1a). Similarly, SOD reached its highest activity level, which was 3.74 times to the control, when BaP concentration was 100 μg L^{-1} in medium, a middle level of concentration in our study (Fig.1b). Growth of *P. tricornutum* exposed to 500 μg L^{-1} DDT was most inhibited and SOD activity was 3.73 times to the control (Fig.1c).

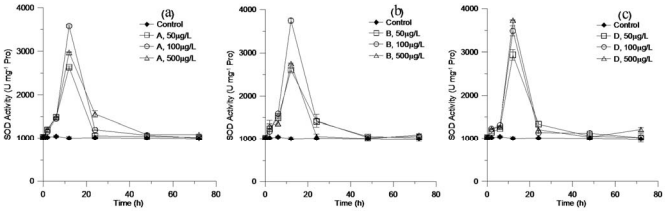

Figure 1. SOD activity in *P. tricornutum* cultured for 72 h in f/2 medium supplemented with 50 μg L^{-1}, 100 μg L^{-1} and 500 μg L^{-1} of Aroclor 1254 (A), BaP (B) and DDT (D). *P. tricornutum* exposed to seawater was used as control. One unit (U) of SOD (per mg protein) was defined as the amount causing 50% inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). Bars represent mean values of three independent replicates ±1 SD.

In *S. costatum* cultivated under the same conditions, changes of SOD activities were similar to those in *P. tricornutum* (Fig.2). Specifically, Aroclor 1254 and BaP with the final concentration of 100 μg L^{-1} in the medium directed to highest SOD activities which were respectively 2.77 and 2.89 times to their controls after 12 h of exposure (Fig2a, b). However, SOD activity reached its peak when 500 μg L^{-1} of DDT was used (Fig.2c), though the SOD activity increase was not as high as those in Aroclor 1254 and BaP added medium. In addition, SOD activities after 12 h of exposure to DDT with concentrations of 50, 100 and 500 μg L^{-1} were 2.75 times, 2.89 times and 2.96 times to the control, respectively (Fig.2c). Statistical results showed that algae exposed to DDT were significantly inhibited (P<0.05) but there was no significant difference among the three concentrations (P>0.05). For both *P. tricornutum* and *S. costatum*, SOD activities declined gradually when exposed to all of the three POPs, and reached the stable basal levels which were not significantly different from the control (P>0.05).

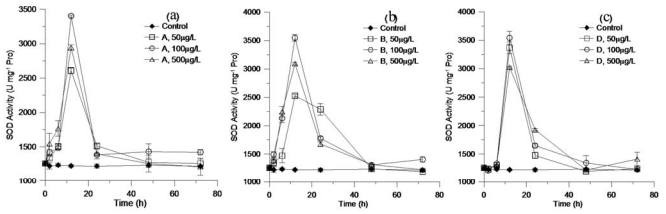

Figure 2. SOD activity in *S. costatum* cultured for 72 h in f/2 medium supplemented with 50 μg L^{-1}, 100 μg L^{-1} and 500 μg L^{-1} of Aroclor 1254 (A), BaP (B) and DDT (D). *S. costatum* exposed to seawater was used as control. One unit (U) of SOD (per mg protein) was defined as the amount causing 50% inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). Bars represent mean values of three independent replicates ±1 SD.

Detection of MDA content

The effect of the POPs, including Aroclor 1254, BaP and DDT, on MDA production in *P. tricornutum* was obvious after 2 h of exposure, although the kinetics varied depending on the pollutant concentration (Fig.3). In *P. tricornutum* the maximum MDA level was detected after 2 h of exposure, which was 2.63-3.29 times to the control, and then followed by rapid decay to reach the control level (P>0.05) (Fig.3). The change trends of MDA concentrations were similar to those of SOD activities when *P. tricornutum* was treated with different concentrations of POPs, except for the earlier peaking. Similarly, the MDA concentration peak appeared when 100 μg L^{-1} of Aroclor 1254, 100 μg L^{-1} of BaP and 500 μg L^{-1} of DDT were used.

For *S. costatum* cultivated under the same conditions, the MDA concentrations peaked after 2 h of exposure and were 1.84-2.40 times to the controls (Fig.4). Differently, all the highest MDA levels came from the treatment of 500 μg L^{-1} of the three POPs, though varied pollutant concentrations did not result in an obvious effect on highest MDA concentrations, especially when 100 and 500 μg L^{-1} of pollutants were used.

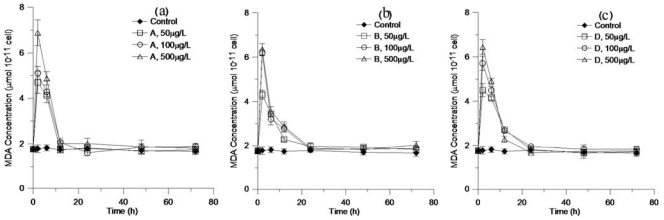

Figure 3. MDA concentrations in *P. tricornutum* cultured for 72 h in f/2 medium supplemented with 50 μg L^{-1}, 100 μg L^{-1} and 500 μg L^{-1} of Aroclor 1254 (A), BaP (B) and DDT (D). *P. tricornutum* exposed to seawater was used as control. Bars represent mean values of three independent replicates ±1 SD.

Effects of POPs on growth behavior of P. tricornutum *and* S. costatum *in mixed-culture*

When the initial cell densities of both *P. tricornutum* and *S. costatum* were 2.5×10^5 cell mL^{-1}, growth of *P. tricornutum* in bi-algal cultures was totally inhibited under the stress of the different concentrations of the three POPs during the 28-day experimental period (Fig.5a). However， it is important to mention that under all these conditions, cell densities of *P. tricornutum* increased up until the end of the experiment though its growth was inhibited by the POPs. In contrast, *S. costatum* reached the maximum cell densities at day 10 and then decreased immediately. Its growth was enhanced by POPs after 19-day of exposure to 50 μg L^{-1} Aroclor 1254, 50 μg L^{-1} BaP and 15 μg L^{-1} DDT (Fig.5d).

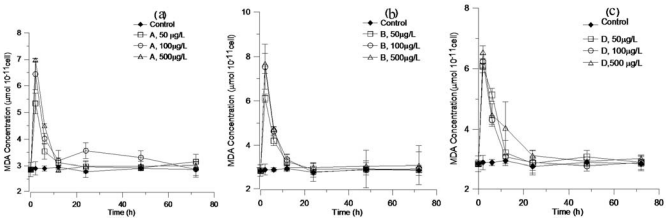

Figure 4. MDA concentrations in *S. costatum* cultured for 72 h in f/2 medium supplemented with 50 μg L^{-1}, 100 μg L^{-1} and 500 μg L^{-1} of Aroclor 1254 (A), BaP (B) and DDT (D). *S. costatum* exposed to seawater was used as control. Bars represent mean values of three independent replicates ±1 SD.

When the initial cell densities of *P. tricornutum* and *S. costatum* were 1×10^5 and 4×10^5 cell mL^{-1} respectively, growth behavior of *P. tricornutum* in different conditions was almost identical to the control except in the culture under exposure to 50 μg L^{-1} BaP exposure (Fig.5b). Compared to 15 μg L^{-1} BaP, this higher concentration led to an enhanced function on *P. tricornutum* growth during day 10 to day 25 of exposure, wherein the maximum ratio ($ratio_{max}$) of cell density was 128%. In the same medium, results showed that under 15 μg L^{-1} BaP ($ratio_{max}$ = 215%), 15 μg L^{-1} DDT ($ratio_{max}$ = 542%) and 50 μg L^{-1} DDT ($ratio_{max}$ = 494%) exposure, the growth of *S. costatum* also increased significantly compared with the control (Fig.5e). More importantly, the growth of *S. costatum* was strongly enhanced by Aroclor 1254, BaP, and DDT in the bi-algal culture that cell densities at day 28 were almost the same as the maximum levels of the control culture. That is to say, the presence of POPs caused abnormal growth of *S. costatum* in the mix-culture.

When the initial cell densities of *P. tricornutum* and *S. costatum* were 4×10^5 and 1×10^5 cell mL^{-1}, the growth of *P. tricornutum* increased significantly in medium enriched with 50 μg L^{-1} DDT ($ratio_{max}$ = 148%) during the 28 days experiment. In contrast, cell densities of *S. costatum* increased under exposure to 50 μg L^{-1} BaP ($ratio_{max}$ = 212%, day 10-28) and 50 μg L^{-1} Aroclor 1254 ($ratio_{max}$ = 139%, day 10-22).

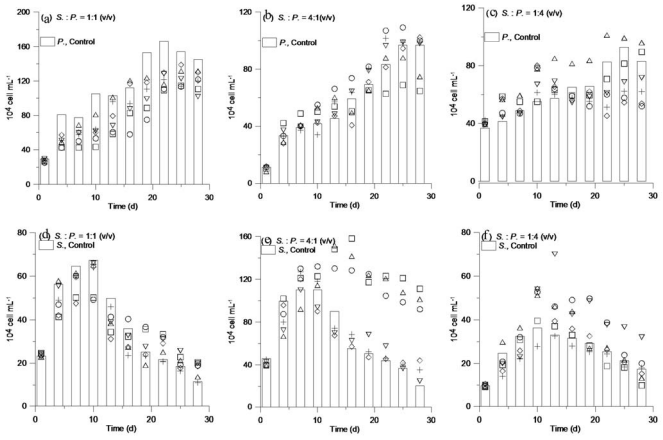

Figure 5. Growth of *S. costatum (S.)* cultured with *P. tricornutum (P.)* for 28 days. Initial cell density of both *S.* and *P.* were 2.5×10^5 cells mL^{-1} (a, d); Initial cell density of *S.* was 4×10^5 cells mL^{-1} and *P.* was 1×10^5 cells mL^{-1} (b, e); Initial cell density of *P.* was 4×10^5 cells mL^{-1} and *S.* was 1×10^5 cells mL^{-1}. A = Aroclor 1254, B = BaP, D = DDT. Control stands for the algal culture exposed to control seawater.

+, 15 μg L-1 A; ◇, 15 μg L^{-1} B; □, 15 μg L^{-1} D; ▽, 50 μg L^{-1} A; ○, 50 μg L^{-1} B; △, 50 μg L^{-1} D.

IV. DISCUSSION

This study provides strong evidence showing that Aroclor 1254, BaP and DDT cause stress condition in growth of both *P. tricornutum* and *S. costatum*. They showed a good dose-response at different levels of POPs. It has been reported that antioxidant system such as POD, SOD and other antioxidant compounds that provide antioxidant protection in algae increased on the first day of exposure to pollutants[20-21]. Therefore, in our study, four time points in the first day (2 h, 6 h, 12 h and 24 h) were selected for investigating the responses of antioxidant systems induced by the chosen POPs. The MDA concentration was clearly demonstrated by the oxidative burst that emerged shortly after the algae were exposed to the three POPs excess, achieving maximum levels after 2 h of exposure, followed by the activities of SOD reached the peak after 12 h of exposure. The obvious result due to the increase in level of ROS in the algae cells indicated that both *P. tricornutum* and *S. costatum* were sensitive to the three chosen POPs. It supported previous reports that microalgae responded rapidly to pollutants because of their small size and high surface volume ratio[22-24].

The dose-response curves for SOD activities exhibited similar shapes for both *P. tricornutum* and *S. costatum* in all the exposure culture experiments (Figs.1, 2). It should be pointed out that the increments did not always raise in sequence with the increase of POPs concentration. 100 μg L^{-1} of pollutants pushed the algae to express the highest SOD activity, but a higher concentration (500 μg L^{-1}) of pollutants resulted in a decreased SOD activity. The recalcitrance of antioxidant enzyme was not surprising considering that the same phenomena had been observed with copper[21], and endosulfan [25] in aquatic microcosms. The damage to algae cells caused by pollutants indicates that increased activity of antioxidant enzymes could reduce oxidative stress in cell [26]. In fact, 500 μg L^{-1} POPs proved to be fatal for *P. tricornutum* and *S. costatum* because SOD activities did not increase with this increased concentration of POPs.

In regard to the response to the presence of excessive POPs, the peak of SOD activity (after 12 h of exposure) appeared later than the peak of MDA concentration (after 2 h of exposure). One possible reason is that SOD activity may be inhibited by ROS at the beginning of POPs exposure[27]. In addition, toxicological mechanisms acting on microalgae may be different among the different marine pollutants. In our study SOD activity levels decreased rapidly after 12 h to 24 h of exposure and reached the control level. This differs from previous results obtained when using fluoranthene on *P. tricornutum* and *S. costatum*[28], wherein there were no significant effects of fluoranthene on SOD activity in both *P. tricornutum* and *S. costatum*. Possibly SOD activity is more sensitive to POPs used in our study (Aroclor 1254, BaP and DDT) than to fluoranthene. Furthermore, antioxidant enzymes did not seem to be of the same importance in conferring tolerance to algae against pollutant-induced stress. In the case of POD activity, statistical results showed that there are no significant differences between the two algal species when exposed to different concentrations (data not shown). The difference between kinetics of SOD and POD showed that SOD was more sensitive than POD to Aroclor 1254, BaP and

DDT in *P. tricornutum* and *S. costatum*. Identifying the sensitive compounds of POPs and studying their effects on algae could provide an enhanced theoretical basis for aquatic ecotoxicology and increase ecological relevance in environmental risk assessment of pollutants[29].

Many previous studies were limited to data on the growth behavior, enzyme responses and gene regulation of single microalgae exposed to excessive pollutants in the environment [15,24,30]. Simple detection of biochemical changes in algae and single alga growth behavior does not provide sufficient information on the toxicological effects of the POPs. As many different species exist in synergy in the natural phytoplankton communities, it is necessary to study the effect of POPs exposure on the whole community. It is necessary to mimic the natural community environment and research algae growth in mixed culture when exposed to pollutants. Therefore we designed bi-algal culture experiments to obtain information about the behavior of both *P. tricornutum* and *S. costatum* to POPs exposure. The results indicated that interaction between *P. tricornutum* and *S. costatum* must also be considered in evaluating the effects of the pollutants in the natural environment. These interactions could not be predicted from the knowledge of the isolated behavior of each algal to single pollutant exposure. Our results of SOD activity and MDA content when exposed to the lowest concentration of POPs in the experiments (50 μg L^{-1}) showed that both *P. tricornutum* and *S. costatum* were under oxidative stress at different levels. The results of bi-algal culture showed that 50 μg L^{-1} DDT could accelerate the growth of the *P. tricornutum* or *S. costatum* in mixed culture of different initial cell densities. More importantly, significant increase of pollutants did not result in an immediate effect on algal growth. The effects took hold a few days after exposure. A previous study on using *P. tricornutum* to assess the toxicity of pyrene provides an explanation for our results. Pyrene only affected actively growing cells [31-32]. According to the phenomena observed in our experiments, the fast growth of algae occurred after 7 days in mixed-culture. The algae was affected by the POPs and showed compensatory growth responses resulting in abnormal incensement. Nevertheless, in order to determine which mechanism is responsible for the dissimilarity between the experimental mix-culture groups, and which mechanism is responsible for the toxicity intensity between the same pollutants, further research needs to be conducted.

In the future, it will still be a challenge to determine the mechanism by which Aroclor 1254, BaP and DDT affects *P. tricornutum* and *S. costatum*. Also the combination effect of POPs on microalgae needs to be studied in greater detail.. Due to their harmful nature POPs warrant continuous environmental monitoring. The frequent testing of aquatic organisms especially algae will assist in this monitoring process. It is also necessary to investigate the interaction between different phytoplankton species under the exposure of mixed persistent organic pollutants.

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