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Effects of tributyltin on the chlorophyll contents of marine microalga *Tetraselmis tetrathele, Nannochloropsis oculata* and *Dunaliella* sp.

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Abstract—Three species of marine microalga *Tetraselmis tetrathele, Nannochloropsis oculata* and *Dunaliella* sp., the important phytoplankton in many hatcheries, were used in an acute toxicity test of TBT with emphasis on the chlorophyll a and b contents. After a very short exposure time of three concentrations of TBT, 0.1, 0.5 and 1 $\mu g\,l^{-1}$, the three species of algae showed different responses. The chlorophyll a and b contents of *T. tetrathele* in all treatments were higher than those in controls, even be doubled in 1 $\mu g\,l^{-1}$ of TBT. *N. oculata* and *Dunaliella* had a slightly higher chlorophyll a and b contents in the lowest TBT concentration tested (0.1 $\mu g\,l^{-1}$) than those in control, but as the TBT concentration increased their chlorophyll contents decreased. Three levels of TBT tested are within the range of the no observable effect concentration (NOEC) for *T. tetrathele*, while the lowest observable effect concentrations (LOEC) for *N. oculata* and *Dunaliella* are between 0.1 to 0.5 $\mu g\,l^{-1}$. Among the three species, *N. oculata* has a highest sensitivity towards TBT.

Key words: tributyltin, marine microalga, chlorophyll a, chlorophyll b

Introduction

TBT is one of the organotin compounds used extensively as an active biocide and plastic stabilizer around the world (Mercier et al. 1994, EPA 2003). The environmental concern about the utilization of this compounds has increased remarkably in the last 20 years (Morabito and Quevauviller 2002). It is worldwide recognized as the most dangerous chemical ever introduced in large quantities in estuaries and coastal waters (Bekri and Pelletier 2004, Harino et al. 1998, 2003), even more toxic than the other biocides such as chlorothalonil, irgarol, diuron, and dichlofluanid (Fernández-Alba et al. 2002). The consequence of the direct introduction into the marine environment and widespread of its toxicity to non-target aquatic animals such as mussels, clams, oysters, seastar and crustaceans has been well documented (Morabito and Quevauviller 2002, Ohji et al. 2002a, b, EPA 2003). In addition, as a lipophilic compound, TBT tends to accumulate in aquatic plants as well, including phytoplankton.

Phytoplanktons are dominated by microalgae, which play a role in both primary production and nutrient recycling in marine ecosystem. Therefore, any adverse effects of pollutants on microalgae may lead to serious ecological consequences. In addition, if xenobiotic are bioconcentrated by microalgae, this may potentially lead to food chain transfer or biomagnification (Yang et al. 2002). Measuring the relative and absolute concentration of photosynthetic pigments is important in assessing the relationship of microalgae to their environment in particular chlorophyll contents (Rowan 1989).

There is a paucy of data on toxicity effects of TBT on marine microalgae compared to other aquatic organisms (EPA 2003). The biological significance of TBT effect on phytoplankton, however, would be a basic estimate for assessing aquatic food web. Among trophic levels in the aquatic ecosystem, microalgae are highly sensitive even to low doses of toxic chemicals including TBT (Fernández-Alba et al. 2002). Despite this, limited studies have been conducted with Chlorophyta, especially with respect in photosynthetic pigments.

An initiative study on the TBT toxicity effect of TBT was conducted by employing three representative species of microalgae with respect in their chlorophyll contents. As

main photosynthetic pigment, chlorophyll a is ubiquitous in algae, and chlorophyll b as an accessory pigment, occurs in all classes of Chlorophyta (Rowan 1988). The three species used belong to Chlorophyta. The method used was a static type because of the very short exposure time with a low loading (biomass/volume of water) of test cells. This system is generally the only one that can be used if a very limited quantity of the chemicals to be tested is available (Van Leeuwen 1988).

Materials and Methods

Toxic effects of TBT has been examined on change of the chlorophyll a and b contents of TBT-exposed marine microalgae *Tetraselmis tetrathele*, *Nannochloropsis oculata* and *Dunaliella* sp. The algal cells were cultured in diluted seawater (30 g l^{-1}) in 2 l-glass containers containing Hirata medium $(NH_4)_2SO_4$, $Na_2HPO_4 \cdot 12H_2O$ and the commercially available concentrated nutrients, Clewat 32, with the concentrations of 122.6, 23, and 15 mg l^{-1}, respectively (Hirata 1975). The culture chambers were incubated at 25–27°C under continuous illumination. From the exponential phase of each culture stock, where the cell densities reach 10^6 cells ml^{-1} for *N. oculata* and *Dunaliella* sp., and 10^5 cells ml^{-1} for *T. tetrathele*, a two hundred ml-algal suspension was transferred into each of four flasks for three treatments and a control. The treatments were three TBT concentrations, 0.1, 0.5 and 1.0 $\mu g\, l^{-1}$ exposed for a very short of time (6 hours).

For chlorophyll measurements of all treatments with two replicates, cells were extracted in 90% acetone and debris were removed by centrifugation at 3000 rpm for 15 min. The absorbance of the supernatant at 664 nm and 645 nm was measured with DR/3000 spectrophotometer. The Chlorophyll a and b concentrations of the samples were determined according to the common equation (Mackinney 1941).

Results and Discussion

The responses to TBT exposure with respect in chlorophyll production were different in three species of microalgae tested. Table 1 shows the magnitude of chlorophyll contents of each species. For all treatments, the magnitude of chlorophyll a contents of *T. tetrathele* and *N. oculata* were all higher than the chlorophyll b ones, only *Dunaliella* sp. had similar range of values for the two types of chlorophyll, all values were below 5 $\mu g\, l^{-1}$. The tendency of change in the values as TBT concentration increase is similar for both chlorophyll a and b contents. This may associate with the basic relationship between them, that the energy of light transferred from chlorophyll b to chlorophyll a during photosynthesis (Rowan 1988).

For more explanation, the magnitude of chlorophyll contents of each species was then transformed into relative values, by dividing each respective real value by that of the respective control. Therefore, the relative values of control are 100% or 1. As shown in Fig. 1, the three species have different sensitivities to different TBT concentrations. For *T. tetrathele*, exposure to TBT resulted in increase of chlorophyll contents, even up to 210% and 252% at highest concentration of TBT (1.0 $\mu g\, l^{-1}$) for chlorophyll a and b, respectively. However, for the other two algae, *N. oculata* and *Dunaliella* sp., the elevated chlorophyll a and b contents occurred only at the lowest concentration tested (0.1 $\mu g\, l^{-1}$), afterward the chlorophyll contents decreased sharply as TBT concentration increased. The chlorophyll a and b contents of *T. tetrathele* in all treatments were higher than those in controls, even be doubled in 1 $\mu g\, l^{-1}$ of TBT. *N. oculata* and *Dunaliella* had a slightly higher chlorophyll a and b contents in the lowest TBT concentration tested (0.1 $\mu g\, l^{-1}$) than those in control, but as the TBT concentration increased their chlorophyll contents decreased.

Within these very few data, all concentrations of TBT tested had no adverse effects on *T. tetrathele* when exposed to a very short time, but for the other two species the concen-

Table 1. Chlorophyll a and b contents ($\mu g\, l^{-1}$) of *T. tetrathele*, *N. oculata* and *Dunaliella* sp. exposed to different TBT concentrations.

Microalga	TBT concentration ($\mu g\, l^{-1}$)							
	0.0		0.1		0.5		1.0	
	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b
T. tetrathele	12.58	5.68	21.05	11.31	22.51	11.72	26.71	14.35
N. oculata	24.42	10.12	25.21	10.79	20.62	7.52	18.32	6.44
Dunaliella sp.	2.58	2.21	4.64	4.57	2.78	1.92	2.39	1.81

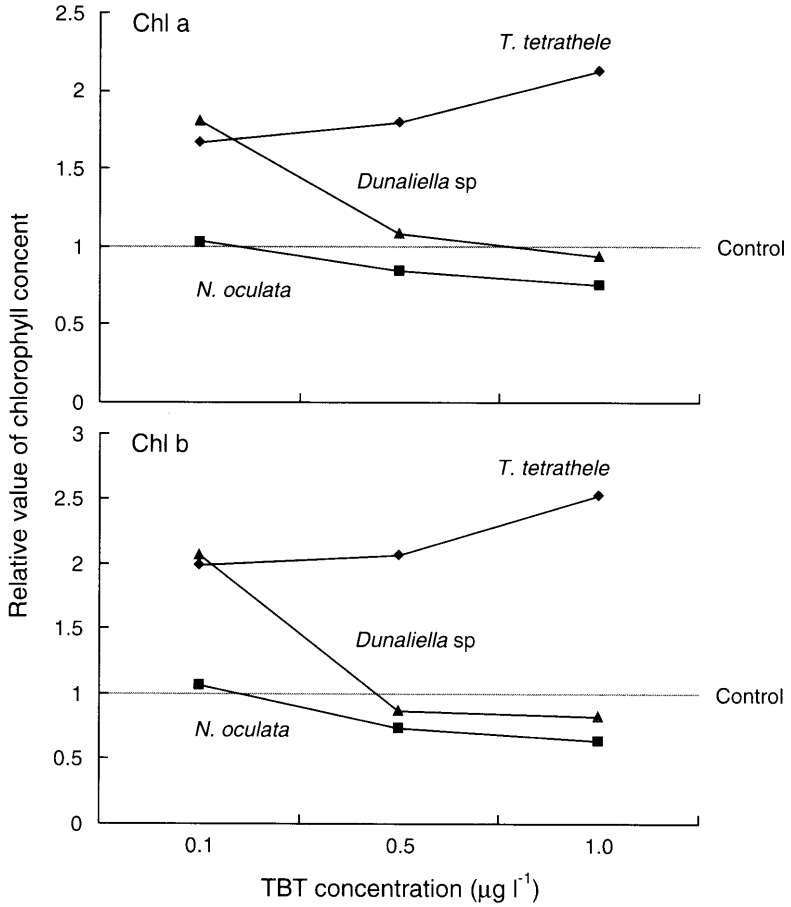

Fig. 1. Relative values of chlorophyll contents of microalgae exposed to different concentrations of TBT ($\mu g\,l^{-1}$). Dash line shows the value of control, which is equal to one (1).

traion of $0.5\,\mu g\,l^{-1}$ had been critical level with respect in chlorophyll synthesis. It is obvious that *T. tetraselmis* had more wide range of sublethal concentration of TBT than those *N. oculata* and *Dunaliella* had during the short period of exposure time (6 h). Longer time of TBT exposure caused the change in color of the algal suspension in the preliminary experiments. The unexpected response of *T. tetrathele* to all concentrations tested suggest that a certain range of toxicant concentrations may induce organisms to react by increasing a certain metabolic process during the very short exposure time. We see such a strategy in zooplankton, for example rotifers, *Brachionus plicatilis*, as reported by Cochrane et al. 1991. They demonstrated that exposed to sub-lethal concentration of some toxicants including TBT, the rotifers produced more stress proteins, even 4–5 fold increase in heat shocked proteins. This phenomenon should be more clarified for microalgae to prove that if there is a species–specific typical strategy to adapt in unfavorable environmental condition for plankton. Further work is obviously required to examine on how they react toward a stressor under a certain degree of environmental condition. It is different from the degree to which an organism could survive, or the EC_{50}.

The sensitivity of the bioassays should be evaluated by comparing the lowest observable effect concentration (LOEC) (EPA 2003). Most antifouling compounds have the LOEC values of ppb levels, which represents the initial toxicity threshold of a chemical (Fernández-Alba et al. 2002). It is necessary to determine first not only LOEC but also the no observable effect concentration (NOEC) in oder to develop a standardized acute bioassay and LC_{50} values for a variety of reference toxicants including TBT. Very few data of phytoplankton are available in the list of aquatic organisms exposed to TBT as reviewed by US EPA (2003), among them are the list of TBT concentrations including EC_{50} that had significant effects on growth of *Dunaliella*, from 1 to $6\,\mu g\,l^{-1}$. The only one for *T. tetrathele* is the EC_{50} ($6.06\,\mu g\,l^{-1}$) in term of reduced growth after 7 days. More information for common unicellular algae that the 72-hour EC_{50} ranges from 0.33 to $1.03\,\mu g\,l^{-1}$ of tributyltinoxide (TBTO) (Walsh 1986). It can be concluded in this study that the three levels of TBT tested

are still in range of NOEC for *T. tetrathele*, and the LOEC for *N. oculata* and *Dunaliella* are between 0.1 to 0.5 $\mu g\,l^{-1}$.

The adverse toxic effects occurred at different concentrations of TBT when tested using the different taxa. For example, the toxic level of TBT for microalga *Selenastrum capricornotum* was seen at concentration of 0.1 $\mu g\,l^{-1}$, much lower than the toxic level for microscopic crustacean *Daphnia magna* (0.8 $ng\,l^{-1}$) (Fernández-Alba et al. 2002). The most adverse effects are corresponding to acute and chronic values. No information available on those values for phytoplankton in the list provided by EPA (2003). Since acute toxicity tests are the first steps toward understanding the toxic effects of materials in the aquatic system (Van Leeuwen 1988), the next step is chronic tests would be possible, for *N. oculata* and *Dunaliella* sp. by applying lower concentration than 0.5 $\mu g\,l^{-1}$ to provide a reference point closer to the actual no-effect level at the ecosystem level. For *T. tetrathele*, it needs more acute toxicity tests to find the LOEC. Both acute and chronic toxicity of TBT to a wide variety of phytoplankton are obviously necessary to investigate not only in term of chlorophyll synthesis, but also on other biological parameters. This study is only first step toward characterization of the effects of TBT exposure on pigment synthesis. Furthermore, as the concentration of TBT, in combination with the duration of exposure, is of prime importance for determining whether adverse effect of TBT, it is required to assay more range of concentrations of TBT with different exposure time, with the design and calibration of an efficient assay system. A possible alternative assay that would allow detection of effects of shorter exposure may be to assay cellular structures.

More over, one of the most significant aspects of TBT toxicity to be concerned is its biodegradation and biotransformation, and succesive biomagnification. As a chronically and acutely toxic compound, TBTs half-life in aerobic and anaerobic conditions ranges from 6 to 9 months and its degradation products are also toxic and persistent (Fernández-Alba et al. 2002). The bioaccumulation and biotransformation of xenobiotics such as 2,4-dichlorophenol have been studied extensively in certain diatom (Yang et al. 2002). No data of such a study is currently available on other of phytoplankton. The three microalgal species tested were selected because their representatives of all major phytoplankton that used as live preys. These microalgae have been extensively used as a major feed source for the initial stage of aquatic fauna and zooplanktonic live preys. During the last decade, we have used these algae for feeding the local rotifers and copepods for studies. However, from the ecological point of view, if these microalgae have high vulnerability to xenobiotic bioaccumulation, they might play a role as "toxicant-transferring organisms" into higher rank in trophic level such as zooplankton and fish larvae. Fernández-Alba et al. (2002) found that a certain freshwater green microalga (*Selenanstrum capricornotum*) was the most sensitive towards the biocides including TBT compared to other taxa such as representative cladocerans (*Daphnia magna*) and bacterium (*Vibrio fischeri*). Trophic transfer via live prey to higher taxa has also been recently investigated by Bekri and Pelletier (2004) for seastar. This suggests that possible transferring via microalgal might occur in field. Therefore, for assessing marine ecosystem, further researches on biodegradation, biotransformation and biomagnification of TBT in microalgae are necessary. This could provide significant information on microalgae as field bioindicator of spatial and temporal trends of this pollutant.

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