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Effects of tributyl-tin on a marine microalga, *Tetraselimis suecica*

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Abstract: *Marine pollutants induce changes in microalgal metabolism. In this study effects of tributyl-tin chloride (TBTCl) on a marine microalga Tetraselmis suecica was studied. The changes induced by TBTCl on growth rate, viability and biochemicals were assessed. In acute exposure to TBTCl, EC_{50} estimated for 24 hr was 2.02 µg ml^{-1}, whereas total lethality was observed at 4 µg ml^{-1}. In chronic exposure to TBTCl, at higher concentrations (0.5-1 µg ml^{-1}) growth rate, chlorophyll pigments, carbohydrate and protein contents were reduced. The results of this study indicate that TBTCl toxicity made drastic changes in growth and biochemical composition of T. suecica.*

Key words: *TBT toxicity, Growth performance, Biochemical composition, Tetraselmis suecica*
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Introduction

Tributyl-tin (TBT) copolymer based antifouling paint releases very small amounts of TBT due to which continuous leaching of TBT is maintained on the ship hull surface (usually at the rate of 1.5 µg cm^{-2} d^{-1}) thus avoiding biofouling problem. The broad spectrum antifouling (AF) effectiveness of organotin (tributyl-tin, tributyl-tin oxide, triphenyl-tin *etc.*) compounds was recognized in the 1960s and in the 1970s as organotin copolymer paints were largely used at that time. In the late 1970s a link was established between the use of TBT antifoulants and deformities in oysters (Alzieu *et al*., 1986). Organotin leachates from anifouling paints are toxic to marine organisms. Laughlin *et al*. (1982) have shown that TBT leachates from antifouling paint are extremely toxic to nontarget marine amphipods. TBT released from antifouling paint, adversely affects the marine environment thus earning a ban on TBT paint application all over the world (Stewart, 1996).

In the recent past, there has been an increased awareness of the drastic environmental problems caused by the antifouling compounds. Especially, a wide range of negative impacts of TBT have been documented (DeMora, 1996). The effects of organotin compounds on growth and metabolism of microalgae have been well investigated (Wong *et al*., 1982; Walsh *et al*., 1985; Beaumont and Newman, 1986; Huang *et al*., 1993; Sidharthan *et al*., 2002). The response of microalgae to TBT toxicity varies with dose and exposure time. High toxicity of TBT is demonstrated in acute toxicity assays but the effects varied in different species of microalgae. In general, TBT toxicity causes sudden reduction in growth rate and affects the photosynthetic efficiency of microalgal species (Cooney and Wuretz, 1989; Fargosava, 1998). Moreover microalgae have been reported to accumulate low concentrations of TBT (Maguire *et al*., 1984; Chiles *et al*., 1989; Huang *et al*., 1993) which is close to environmental concentrations reported from many coastal regions around the world. However, microalgae have the ability to degrade TBT (Lee *et al*., 1989; Saint-Louis *et al*., 1994; Tam *et al*., 2002). In the marine environment TBT (Bu_3Sn^+) may degrade into less toxic di- butyltin (DBT: Bu_2Sn^{2+}), mono-butyltin (MBT: $BuSn^{3+}$) and tin (Sn: Sn^{4+}) by a sequential debutylation mechanism (Gadd, 2000; Tam *et al*., 2002; Saeki *et al.,* 2007).

The range of TBT concentrations in seawater reported from Asian countries such as Taiwan (ND–229.4 ng l^{-1}, Lee *et al*., 2006), India (123–345 ng l^{-1}, Bhosle *et al*., 2004), Japan (8.2–12.9 ng l^{-1}, Murai *et al*., 2005) and Korea (ND–7.7 ng Sn l^{-1}, Shim *et al*., 2005) and in other marine environmental compartments (Shin and Sidharthan, 2002; Shim *et al*., 2004) indicate the possibility of adverse effects on microalgae. Therefore, TBT toxicity has a potential environmental risk as it can be bioconcentrated and transferred to food chain in the marine ecosystem. The main objectives of this study were to evaluate the effects of TBT toxicity to a microalga, *Tetraselmis suecica*.

Materials and Methods

The microalga, *Tetraselmis suecica* used in this study was obtained from the Korean Microalgae Culture Center (KMCC), S. Korea. A stock culture of *T. suecica* was maintained in f/2 medium [filtered (0.45 µm) seawater l^{-1}: 75 mg of $NaNO_3$, 5 mg of NaH_2PO_4 H_2O, 30 mg of Na_2SiO_3 $9H_2O$, 315 mg of $FeCl_3$ $6H_2O$, 436 mg of Na_2EDTA $2H_2O$, 9.8 µg of $CuSO_4$ $5H_2O$, 6.3 µg of Na_2MoO_4 $2H_2O$, 22 µg of $ZnSO_4$ $7H_2O$, 10 µg of $CoCl_2$ $6H_2O$, 180 µg of $MnCl_2$ $4H_2O$, 0.5 µg of B_{12}, 0.5 µg of Biotin and 100 µg of Thiamine HCl : salinity 25 psu and pH 7.5] (Guillards and Ryther, 1962). Tributyltin chloride (TBTCl, Aldrich Chemical Company, USA) was dissolved in methanol (0.5 ml) and constituted with f/2 medium to make a stock solution of 1000 mg l^{-1}. From this stock solution, a series of dilutions were made with f/2 medium to get the desired concentrations (0.1-100 µg ml^{-1}). The test concentrations of TBTCl were chosen from the preliminary tests. Both acute (96 hr) and chronic (10 days) toxicity tests were conducted. Acute toxicity tests were conducted with 0.25, 0.5, 1, 2, 3, 4 µg ml^{-1} of TBTCl (tributyltin chloride) in multiwell plate (24 well). Growth of *T. suecica* cultures was estimated

by counting culture samples in a haemocytometer (Reichert: Cambridge Instruments Inc., NY, USA). From the slope of regression equation calculated for the cell density (in the exponential phase) against exposure time, the EC_{50} value at 96 hr was determined.

Batch culture was conducted in 250 ml Erlenmeyer flasks with 100 ml of f/2 medium. In batch cultures, sub lethal concentrations were used (0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 µg ml^{-1}). Effects of chronic TBTCl toxicity on cell viability; chlorophyll a and b, protein and carbohydrate contents were evaluated in batch culture experiments conducted for 10 days. All experiments were carried out in a growth chamber (20±1 ^{0}C) under 55 µ E m^{-2} s^{-1} irradiance with 12:12 hr dark:light cycle.

Cell density of test cultures were determined from the optical density read at 678 nm wavelength (UV/VIS spectrophotometer: Ultraspec 3000, Pharmacia, Cambridge, U.K.) as outlined by Hahm *et al*. (2002). Cell viability was assessed by Evans blue staining method (Bodas *et al*., 1995). Chlorophyll a and b contents were estimated using a formula (Chlorophyll a: 11.93 A_{664} – 1.93 A_{647}; Chlorophyll b: 20.36 A_{647} – 5.50 A_{664}: in µg ml^{-1} of 90% acetone extracts) given by Strickland and Parsons (1972). Protein and carbohydrate contents were estimated using standard methods (Dubois *et al*., 1956; Bradford, 1976). Results expressed were mean ±SD from four replicates.

Results and Discussion

The growth of *T. suecica* increased by 13% after 12 hr. exposure to a low concentration of 0.25 µg ml^{-1} of TBTCl (Fig. 1). Growth stimulation decreased to 7% at 0.5 µg ml^{-1}. But 1 µg ml^{-1} of TBTCl decreased 55% of the growth. A maximum reduction of 70% was observed in cultures exposed to 4 µg ml^{-1} of TBTCl. After 24 hr, *T. suecica* cultures exposed to 0.25 µg ml^{-1} of TBTCl, 3% increase in growth was observed, where as at higher concentrations of 1 and 4 µg ml^{-1}, 69% and 77% decrease in growth was observed. A high concentration of 4 µg ml^{-1} TBTCl inhibited growth of *T. suecica* by 90% after 36 hr exposure time. In 48 hr exposure to low concentration of TBTCl, 7-8%, of growth decreased. At TBTCl concentrations above 1 µg ml^{-1}, 90% of growth was inhibited. After 48 hr a high concentration of 4 µg ml^{-1} completely killed *T. suecica* cells. The 24 hr EC_{50} estimated for *T. suecica* was 2.02 µg ml^{-1} (Fig. 1).

In batch culture experiment conducted with sub lethal concentrations of TBTCl, *T. suecica* cells exposed to 0.0312 µg ml^{-1} for two days 23.5% of viability decreased over control (Fig. 3). At 0.0625 µg ml^{-1} viability decreased more than 50% whereas a high concentration of 1 µg ml^{-1} decreased 73.5% of viability (Fig. 3). In *T. suecica* cells exposed to 0.125 µg ml^{-1} of TBTCl for two days, 52% decrease in viability was observed. After four days exposure to 0.0625 and 0.125 µg ml^{-1}, a minimum of <5% viability decreased. But at 0.5 and 1 µg ml^{-1} levels, the viability decreased to 58 and 83%, respectively. During long exposure time low concentrations also caused significant reduction in viability. *T. suecica* cultures exposed to 0.5% and 1 µg ml^{-1} of TBTCl for 10 days, 55 and 93% viability were decreased, respectively (Fig. 3).

Stimulation in growth was seen from fourth day onwards at 0.0312 µg ml^{-1} (Fig. 2). After two days exposure to 0.0312 µg ml^{-1} of TBTCl, chlorophyll a concentration decreased by 1.3% and at 0.0625 µg ml^{-1} the decrease was 10% over control (Fig. 4). Similarly at 0.125 and 0.5 µg ml^{-1}, 42 and 54% reduction were observed, respectively. A maximum of 91% decrease was seen in 1 µg ml^{-1} of TBTCl. On the other hand a 6.6% increase in chlorophyll b content

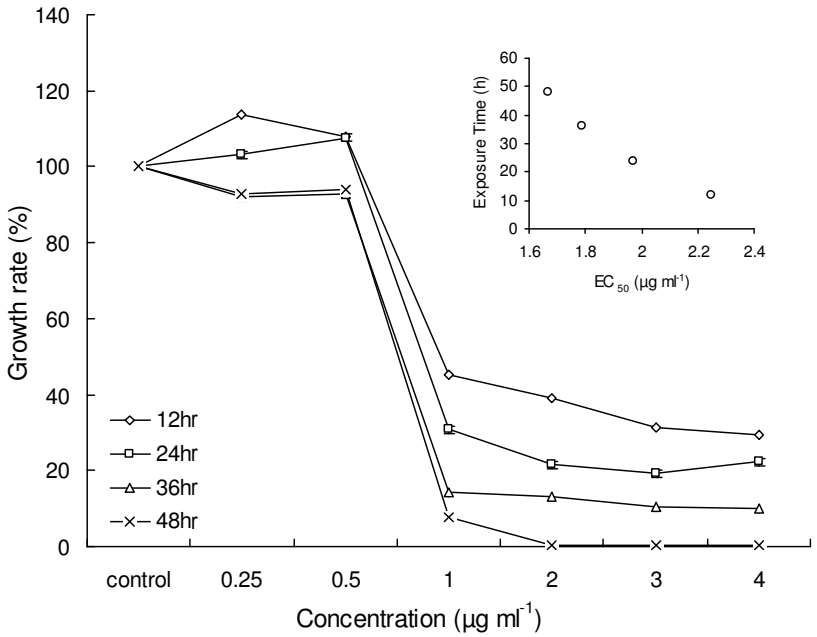

Fig. 1: Acute toxicity of TBTCl to *Tetraselmis suecica* (exposed for 96 hr)

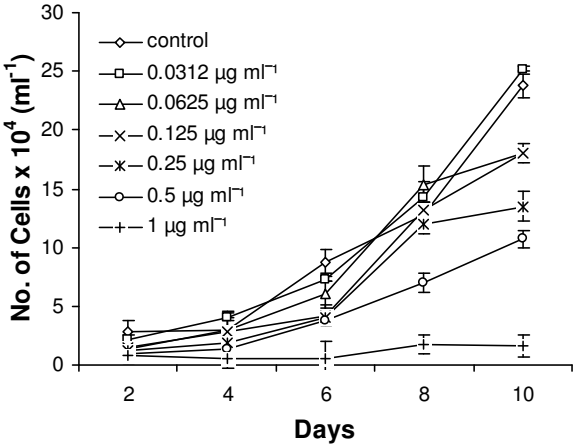

Fig. 2: Effect of TBTCl on growth performance of *Tetraselmis suecica* (exposed for 10 days)

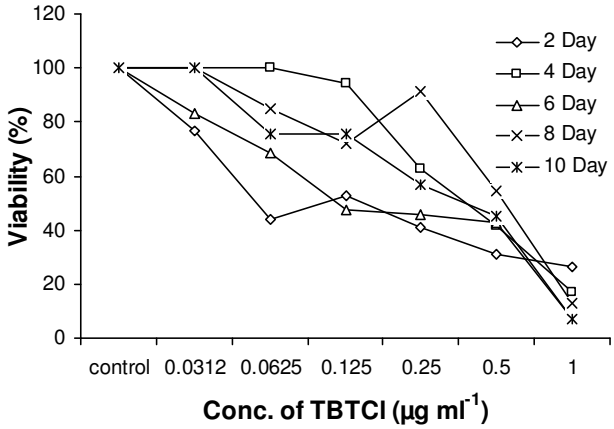

Fig. 3: Effect of TBTCl on viability of *Tetraselmis suecica* (exposed for 10 days)

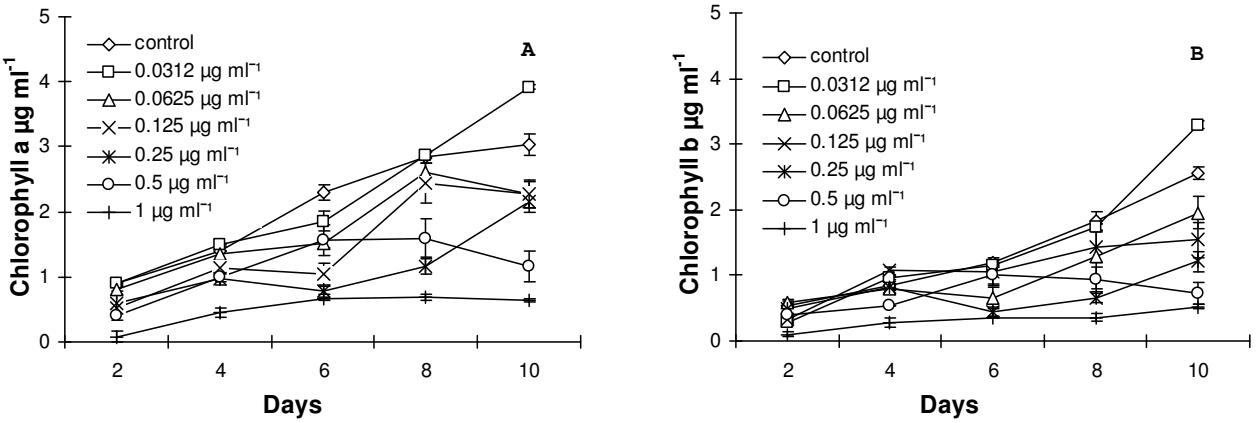

Fig. 4: Effect of TBTCl on chlorophyll pigment contents of *Tetraselmis suecica* (A = Chlorophyll a, B = Chlorophyll b)

was observed at 0.0625 µg ml^{-1}. In TBTCl concentrations 0.125 µg ml^{-1} and above, more than 40% reduction in chlorophyll b was observed (Fig. 4).

After four days exposure time, at 0.0312 µg ml^{-1} of TBTCl increased 13.8% of growth but in 0.5 and 1 µg ml^{-1} levels growth decreased 35.8 and 67.3%, respectively (Fig. 2). At 0.0312 µg ml^{-1}, 6.5% chlorophyll a increased over control. In TBTCl concentration 0.125 and 1 µg ml^{-1}, chlorophyll a contents decreased to 20 and 67%, respectively (Fig. 4). The growth increased to an extent of 54% in 0.125 µg ml^{-1} (Fig. 2). In *T. suecica* cultures exposed to 0.0312 µg ml^{-1} of TBTCl for six days, 19% chlorophyll a content decreased. A high concentration of 1 µg ml^{-1} decreased 71% of the growth. Chlorophyll b concentrations in 0.0312 and 0.25 µg ml^{-1} treated cultures decreased more than 60% (Fig. 4).

After eight days exposure, 0.0312 µg ml^{-1} of TBTCl increases 1.2% of growth. At 0.25 µg ml^{-1}, 51% growth decreased whereas at 1 µg ml^{-1}, 75% growth decreased (Fig. 2). At 0.0625 µg ml^{-1}, 21.2% chlorophyll b decreased whereas a high concentration of 1 µg ml^{-1} decreased 80% (Fig. 4). Increasing culture duration increased the growth of *T. suecica* in lower concentrations of TBTCl (Fig. 2). After 10 days exposure, at a low concentration of 0.0312 µg ml^{-1} 29% chlorophyll a increased (Fig. 4). But at 0.0625 µg ml^{-1} and more chlorophyll a decreased with a maximum of 78% at 1 µg ml^{-1}. At 0.0312 µg ml^{-1}, chlorophyll b increased 28% but at 0.25 and 0.5 µg ml^{-1} it decreased to 52.7 and 71%, respectively.

The carbohydrate and protein concentrations of *T. suecica* cultures varied with TBTCl concentrations administered as well as treatment duration (Fig. 5 and 6). Both carbohydrate and protein contents decreased with concomitant increase in concentrations of TBTCl. However, their concentrations increased with increasing treatment duration, in relation to increase in cell density. More than 80% reductions in carbohydrate and protein contents were observed in *T. suecica* cultures exposed to 1 µg ml^{-1} of TBTCl for 10 days. In *T. suecica* cultures exposed to TBTCl, carbohydrate content was comparatively less inhibited (Fig. 5). A low TBTCl concentration of 0.0312 µg ml^{-1} was found to significantly decrease the protein content of *T. suecica* (Fig. 6). TBTCl was

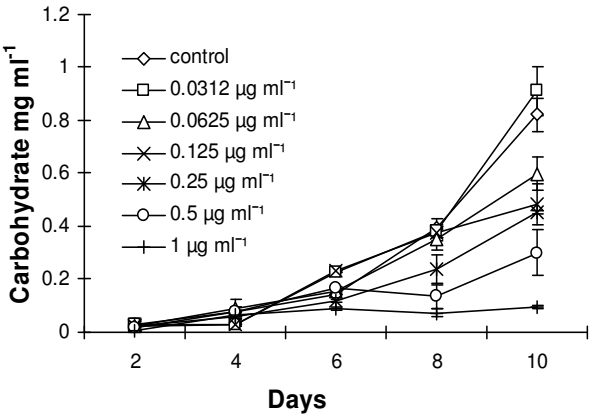

Fig. 5: Effect of TBTCl on chlorophyll pigment contents of *Tetraselmis suecica*

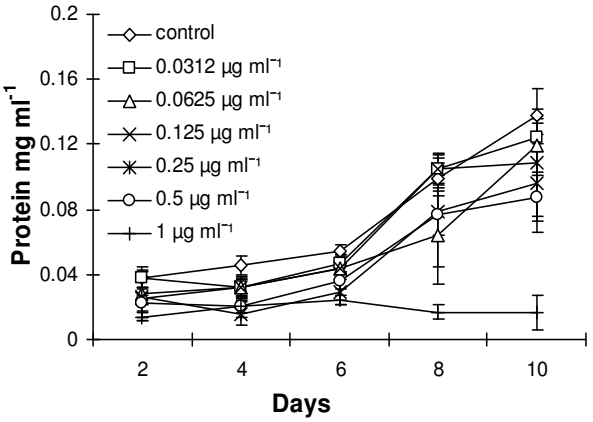

Fig. 6: Effect of TBTCl on protein contents of *Tetraselmis suecica*

found to affect the algal cell metabolisms causing single syllable of cell membranes which leads to adverse changes in the biochemical composition.

The growth of marine fouling organisms on the underwater hulls of vessels causes loss in fuel, speed and economy. Tributyltin based antifouling protection coatings are predominantly used in the shipping industry to avoid troublesome biofouling. TBT leachates from AF coatings are toxic to marine algae. As a consequence, the receiving marine ecosystem is invariably contaminated with TBT. In water column, concentration of TBT is less in the bottom. The decrease in TBT occurs due to biodegradation, sedimentation and volatilization. Diatoms and microalgae seem to promote degradation. However, TBT released into surface waters affects the microalgal productivity (Blanck and Dahl, 1996; Sargian *et al*., 2005; Arrhenius *et al*., 2006). Especially, motile microalgal forms (*T. suecica*) are more prone to TBT exposure.

It has been shown that low concentrations of TBT causes defective shell growth in the oyster, *Crassostrea gigas* (20 ngl^{-1}) (Waldock and Thain, 1983; Evans *et al*., 1995; Swain, 1998). A minium release rate for inhibition of Hydroides elegans larval attachment was found to be 0.5 $\mu g\ cm^{-1}\ d^{-1}$ of TCTCl (Shin and Smith, 2002). The tributyl-tin oxide (TBTO) is well illustrated by its toxic action on wild species of mollusc *Nucella lapillus* (Gibbs *et al*., 1987). Sensitivity of TBTO varies from species to species. TBT is chronically and acutely toxic (Fernández-Alba *et al*., 2002) and inhibits the photosystem II in marine algae. Similarly in the present study also, the photosynthetic pigment concentrations decreased in *T. suecica* cultures as a result of TBTCl induced damage in photosystem.

Wong *et al*. (1982) have shown that trialkyltin compounds are usually more toxic to algal primary production than other forms of organic or inorganic tin. Exposures to 40 and 77 $nmoll^{-1}$ of TPT caused inhibition of growth in *Pavlova lutheri* cultures, whereas slow growth rate inhibition was observed from 23 to 28 $nmoll^{-1}$ of TPT (Marsot *et al*., 1995). Wong *et al*. (1982), reported that low concentrations of 2 to 6 $nmoll^{-1}$ of TPT inhibited the reproduction of microalgae. Millner and Evans (1981) and Callow and Evans (1981), have shown that TPT chloride inhibits phosphorylation in chloroplasts, isolated from macroalga *Enteromorpha intestinalis* and $H_{14}\ CO_3^-$ fixation in *Achnanthes subsessils* respectively.

The EC_{50} (96 hr) obtained for a common biocide coper (I) oxide against *T. suecica* was 1.3 $\mu g\ ml^{-1}$ (Lim *et al.,* 2006). The EC_{50} values of various AF biocides for the microalga, *S. capricornutum* were lower than the EC_{50} values for other organisms, suggesting that the photosynthetic species (*i.e.* phytoplankton) are generally more sensitive to TBT (Fernández-Alba *et al*., 2002). Similarly, 24 hr EC_{50} estimated in the present study for TBTCl against *T. suecica* was found to be less. Beaumont and Newman (1986) used three species of microalgae, commonly used for rearing bivalve larvae, to show that low levels of TBT resulted in reduced growth. It is concluded that even low levels of TBT persisting in the marine environment had significant impact on microalgae. The present study underlines the toxic effects of TBT on marine microalgae.

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