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Toxic effect of triphenyltin chloride on the alga *Spirulina subsalsa*

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A previous study on the deleterious effect of triphenyltin chloride (TPTCl) on the alga *Spirulina subsalsa* reported on four physiological and biochemical indices (or parameters): growth rate, chlorophyll content, phycocyanin content and nitrate reductase activity. In the present study, further research was performed to confirm the findings reported in the previous paper, using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM images show significant changes in the screw-pitch of *S. subsalsa*, suggesting that TPTCl may damage the inheritance characteristics of *S. subsalsa*. The TEM images illustrate that the external pectin theca, limiting membrane and inter photosynthetically active lamella in the *S. subsalsa* cell are those targets that can be easily damaged. Reversible and irreversible cell damage (cell necrosis) are also observed.

KEYWORDS: triphenyltin chloride; *Spirulina subsalsa*; SEM; TEM; toxicity

A variety of organotin compounds have been widely used as marine antifouling paints, agricultural pesticides, wood preservation and plastic stabilizers worldwide. The major sources of organotin compounds to the marine environment are antifouling paints, municipal and industrial wastewater and sewage sludge.[1,2] Tributyltin (TBT) concentrations in sediment are relatively constant with time.[3,4] The half-life of TBT in sediment has been reported to be 2 to 8 years.[5,6] It is also worth mentioning that triphenyltins (TPTs) have a high affinity for particulate matter and tend to enrich in the sediment.[7] TBT and TPT, both used as marine antifouling paints, can directly contaminate aquatic environments, especially the marine environment, due to their long persistence in sediments and their high toxicity. It has been reported that these tin species have high toxicity to a variety of aquatic organisms inhabiting coastal areas, including microalgae, phytoplankton, coastal fish and mussels, invertebrates and benthic organisms. Therefore, among a variety of organotin compounds, TBT and TPT are of most concern to environmental scientists.

Compared with TBT, very little has been reported on the toxicity of TPT to algae, especially to salt-water algae. The fresh-water algae *Senedesmus quadricarda* has been used to study the effect of TPT on organisms.[8–10] However, the fresh-water environment is different from estuarine and ocean environments, so salt-water algae should be used as testing organisms for TPT toxicity in these environments. *Spirulina* spp., which is believed to be a healthy food with the presumed properties of anti-radiation, anti-caducity and anti-mutation,[11] attract the attention of researchers all over the world. However, there are few toxicity tests using *Spirulina* spp. as a testing organism.[12,13] Experiments showed that this alga is easy to culture in salt water and is sensitive to toxicants, so it is a good testing organism for TPT toxicity in estuarine and ocean environments. We have recently reported some preliminary results for the evaluation of toxicity of TPT to *Spirulina subsalsa* using four physiological and biochemical indexes.[14] In the present paper, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and gas chromatography–flame photometric detector (GC–FPD) have been adopted to further study the toxic effects of TPT on *S. subsalsa* in order to elucidate the possible mechanism and degradation pathways of TPT in this alga.

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EXPERIMENTAL

Chemicals and materials

Triphenyltin chloride (TPTcl) was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). Standard TPTcl solution (10^3 μg Sn ml^{-1}) was prepared by dissolving appropriate amounts of TPTcl in ethanol.

The *S. subsalsa* was purchased from the Institute of Aquatic Organism of China. The algae were cultured with Zorrouk solution containing 0.15 mol l^{-1} KNO_3 as nitrate reductone substrate.

Instrumentation

The organotin compounds were analyzed using GC–FPD (HP 58940 A). A fused analytical column with dimensions of 30 m × 0.32 mm i.d., coated with a 0.25 μm film thickness of SE-54 (J & W Scientific) was used. A split/splitless injector was adopted in the split mode; the split ratio was 1:25. The column temperature, injector temperature and detector temperature were maintained at 230 °C, 300 °C and 250 °C respectively. The carrier gas and make-up gas flow rates were 1 ml min^{-1} and 100 ml min^{-1} of nitrogen respectively. The hydrogen gas and oxygen gas flow rates were 100 ml min^{-1} and 20 ml min^{-1} respectively. Injection volume was 1 μl. Data were acquired by Autochro-WIN Chromatography Data (Version 2.0, Young Lin Instrument Co. Korea).

Other instruments used were an Hitachi X-65o scanning electron microscope and an Electron-100 CX transmission electron microscope.

Procedures

Preparation of sample for SEM[15]

Toxicity tests were carried out in 250 ml Erlenmeyer flasks with 60 ml of test solution and the cultures were incubated for 16 h under light (2200 ± 100 lux)/8 h dark photoperiod at 25 ± 1 °C. Each test was done in triplicate. The test lasted for 8 days.

Algal solutions containing TPTCl (10 μg Sn l^{-1}) and control solutions were taken out from the toxicity test, and centrifuged at 3000 rpm for 15 min. The upper clean solution was discarded. The deposits obtained were treated as follows. The deposit was suspended in 5 ml of 2.5% glutaraldehyde aqueous solution; this solution was kept in a refrigerator at 4 °C for 35 h to immobilize the deposit. The immobilized solution was taken out from the refrigerator and centrifuged. The deposit was suspended in a 10% alcohol solution; this was kept in a refrigerator at 4 °C for dehydration; after 40 min, the solution was centrifuged and the supernatant was discarded. The deposit was suspended again in a 20% alcohol solution and kept in the refrigerator for further dehydration; the dehydration was continued in this way with alcohol solutions increasing of concentration gradients. The concentration gradient of alcohol was 10%. Dehydration with each alcohol concentration was conducted for 30 min. Finally, dehydration was carried out twice with absolute (anhydrous) alcohol. The sample was centrifuged; the deposit was collected and was kept in the refrigerator for use. Before observation by SEM, the deposit sample was soaked in 1 ml xylene for 5 min and then coated with gold. The sample was then ready for observation by SEM.

Preparation of sample for TEM[15]

Deposits of algal solutions and control solutions were obtained as described above. The deposits were treated as follows: they were immobilized with 2.5% glutaraldehyde aqueous solution for 2 to 4 h, rinsed several times with fresh buffer solution (phosphate, pH 7.2), then immobilized once again with osmic acid and rinsed with buffer solution (phosphate, pH 7.2) for more than 30 min. Dehydration of the immobilized sample was carried out with an increasing concentration series of alcohol. The concentration sequence of alcohol was 50%, 70%, 80%, 90% and 98%. Dehydration of the immobilized algal sample with each concentration sequence was carried out for 5 to 15 min. Final dehydration was conducted two or three times with absolute alcohol. Each of the final dehydrations was carried out for 5 to 15 min. After final dehydration, the immobilized algal sample was soaked thoroughly in an encapsulating reagent and was encapsulated. Polymerization of the encapsulated sample was carried out at 60 °C for 24 to 36 h. The polymer sample was then prepared as an ultra-thin section using an ultramicrotome; this ultra-thin section was then dyed and observed by TEM.

Preparation of sample for GC–FPD analysis

All algae were separated from the toxicity test water, weighed, homogenized with water and digested with 10 ml of 10% tetramethylammonium hydroxide (TMAH) solution at 60 °C for 1 h. After being cooled, the solution was neutralized with concentrated hydrochloric acid to around pH 1 and was extracted for 30 min with 15 ml 0.3% tropolone in ethyl acetate and hexane (3:2 by volume) after adding 3.0 g NaCl. The organic phase was separated and concentrated with a rotary evaporator, and then derivatized with PeMgBr (Pe = pentyl). The excess Grignard reagent was destroyed with 0.5 M H_2SO_4. The organic phase was separated and evaporated to 0.3 ml under a gentle stream of nitrogen at

Table 1. Retention time of standards and real sample

Compound	TPT standard	DPT standard	MPT standard	Real sample			
Retention time (min)	7.902	4.810	2.923	2.758	3.502 (unknown)	4.718	7.810

Table 2. The effect of TPT on *S. subsalsa* (8 days IC_{50}, $\mu g\ l^{-1}$)

Index	Growth rate	Chlorophyll content	Phycocyanin content	Nitrate reductase activity
TPT	15.63	9.38	31.45	6.05

room temperature. The 0.3 ml solution was kept in the refrigerator for GC–FPD analysis.

RESULTS AND DISCUSSION

It was found that the toxicity of TPT is lower than that of TBT to *S. subsalsa*. Four indices (growth rate, chlorophyll content, phycocyanin content and nitrate reductase) were used to evaluate the effect of TPT on *S. subsalsa*. The results obtained demonstrate that nitrate reductase activity is the most sensitive index to TPT exposure and, therefore, could be used as an important index for evaluation of TPT contamination in the aquatic environment. Because TPT has an inhibitory effect on nitrate reductase activity of *S. subsalsa*, the nitrogen metabolism of algae is changed and consequently the equilibrium of nitrogen metabolism is destroyed, which might influence the nitrogen cycle of the ecosystem.[14]

Degradation of TPT is a process of dealkylation. *S. subsalsa* mainly degraded or metabolized TPT to be less toxic diphenyltin (DPT) and trace amounts of monophenyltin (MPT), which were confirmed by GC–FPD analysis (Table 1). An unknown compound appeared at a retention time between DPT and MPT. We did not identify this compound. From Table 2 it can be seen that, at about 10 ppb (or $\mu g\ l^{-1}$), TPT has a significant toxic effect. Thus 0.5 $\mu g\ l^{-1}$ was selected

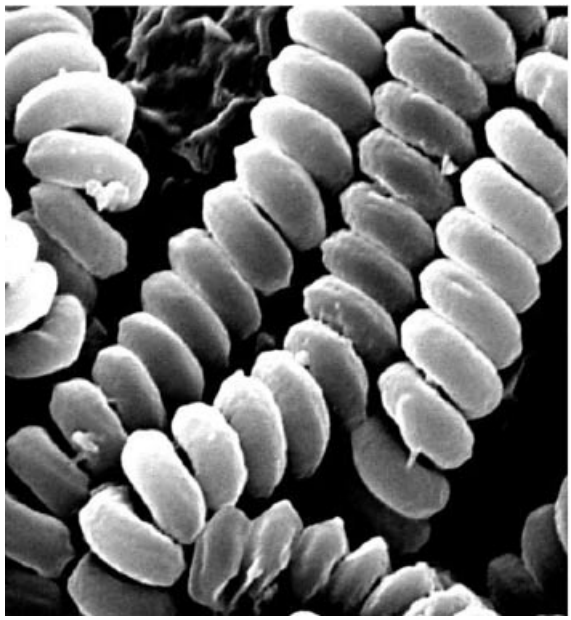

Figure 2. Normal *S. subsalsa* (SEM ×7000).

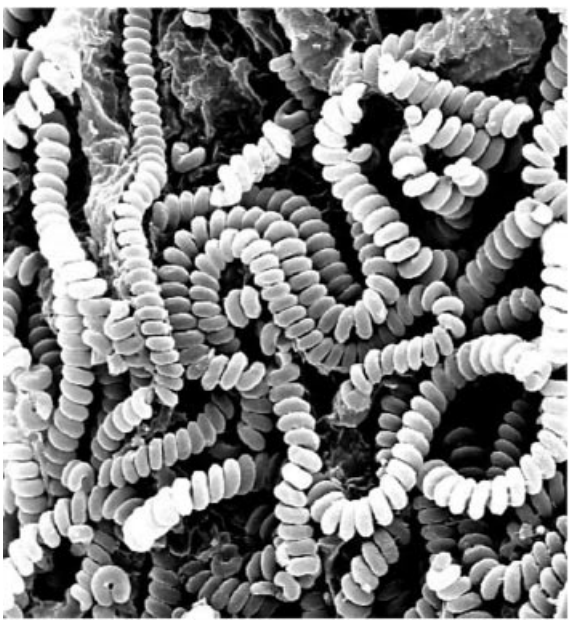

Figure 1. Normal *S. subsalsa* (SEM ×2000).

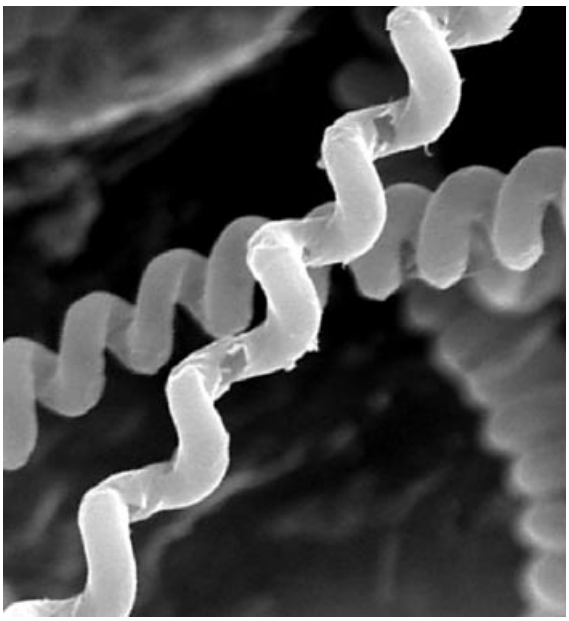

Figure 3. *S. subsalsa* exposed to 0.5 $\mu g\ Sn\ l^{-1}$ TPT (SEM ×7000).

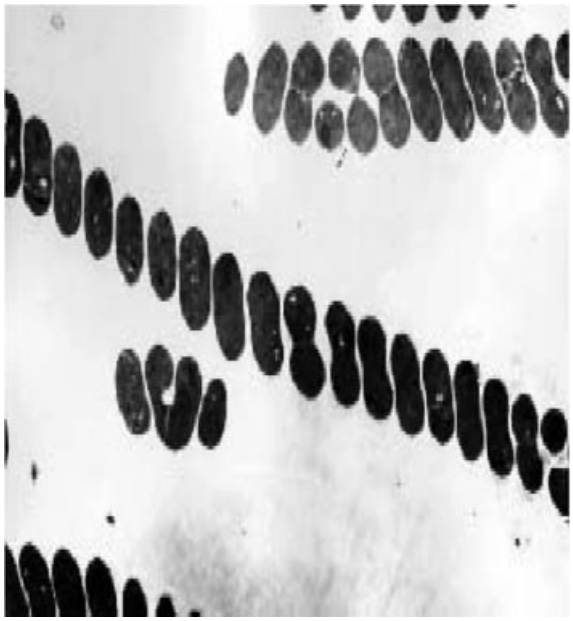

Figure 4. Normal *S. subsalsa* (TEM ×4950).

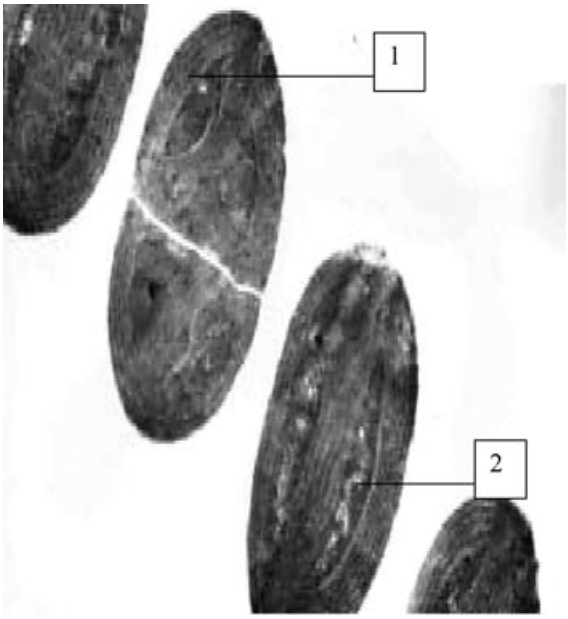

Figure 6. Normal *S. subsalsa* (TEM ×15000) showing the pectin theca.

to carry out the TEM study. This same concentration of TPT was also selected for the SEM study.

The SEM images are shown in Figs 1 to 3. The results of the SEM work showed that TPT changes the screw-pitch length of *S. subsalsa*, which suggests that TPT may have an induced-effect on the inheritance material of *S. subsalsa*. It can be seen from Figs 1 and 2, which illustrate the results of the control group, that *S. subsalsa* has a regular screw-like body shape with a clean smooth surface. The structure of the screw-pitch of *S. subsalsa* is homogeneous and little breakdown of the screw-like algal body is observed. Figure 3 shows the toxic effect of TPT on *S. subsalsa*. A significant change in the screw-pitch appears compared with Figs 1 and 2, suggesting that TPT may change the inheritance characteristics of *S. subsalsa*. Further study is needed to confirm this finding.

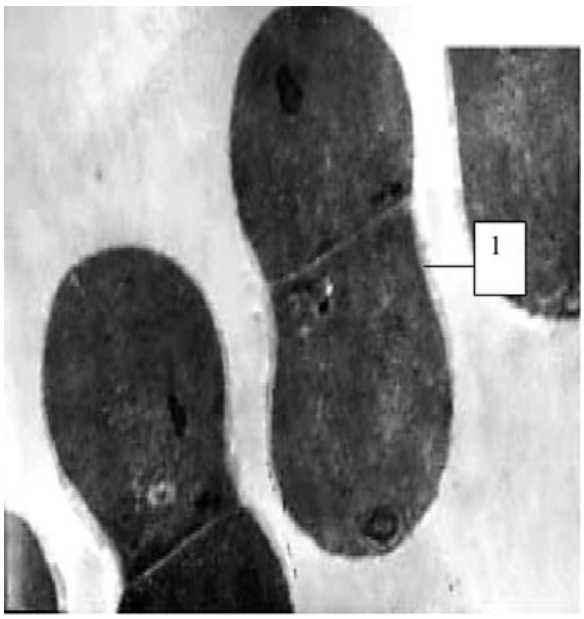

Figure 5. Normal *S. subsalsa* (TEM ×15000) showing the chromogen lamellae and the chromatin apparatus.

Figure 7. *S. subsalsa* exposed to 0.5 μg $Sn l^{-1}$ TPT (TEM ×4950).

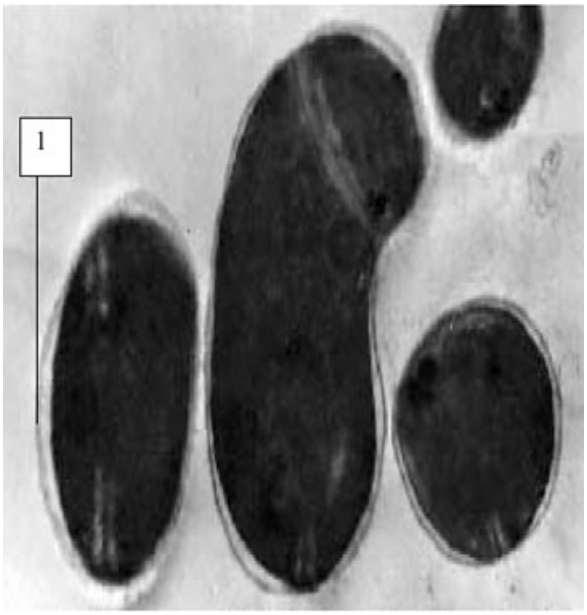

Figure 8. *S. subsalsa* exposed to 0.5 μg $Sn l^{-1}$ TPT (TEM ×12 450) showing pectin theca.

Figure 9. *S. subsalsa* exposed to 0.5 μg $Sn l^{-1}$ TPT (TEM ×15 000).

The TEM images are shown in Figs 4 to 9. Figures 4 to 6 summarize the results obtained under normal experimental conditions (control groups). It can be seen that *S. subsalsa* has a long screw-like body composed of a number of elliptical-type individuals. Without exposure to TPT, the majority of the algae exist as long chains with a small number of algae appearing in the form of a short chain (Fig. 4). The surface of the alga is covered with a pectin theca (Fig. 5). Except for the central region, in most of the algal body there exist chromogen lamellae. These chromogen lamellae, which are parallel to each other, surround the contour of the whole alga body. The lamellae contains phycocyanin, β-carotene, etc. These photosynthetic chromogens are the source of the blue color of *S. subsalsa*. A few gas vesicles with smooth edges are also observed in the cell of the alga. Some particles with different sizes exhibit high electron density within the protoplasm, where the ribosomes are also observed. The lateral and horizontal cell walls can also be observed (Figs 5 and 6). Figures 7 to 9 illustrate the toxic effects of TPT on *S. subsalsa*. The numbers of the long screw-like bodies of *S. subsalsa* are substantially reduced, whereas the numbers of the short ones are clearly increased (Fig. 7). The pectin theca of the algal body swells and eventually dissolves (Fig. 8). Bands with different thicknesses appear along the lateral cell wall. Part of the protoplasm in the algal body undergoes necrosis and collapses. Algal tissue dissolves and disappears. Irregular caverns appear and mylin figures can be seen in the caverns. Gas vesicles increase with irregular edges in the algal body (Fig. 9). In short, observations using TEM illustrate that the external pectin theca, limiting membrane and internal photosynthetically active lamellae of *S. subsalsa* are destroyed by TPT. The TPT also slightly damages the karyoplasm. Reversible and irreversible cell damage (cell necrosis) can be found in the algal body. To our knowledge, this is the first report on the observation of the effects of TPT on *S. subsalsa* using SEM and TEM techniques.

It has been reported that free radicals cause damage to the DNA of plant and animal tissue and in human organs such as the brain.[16–21] The occurrence of mylin figures has been found under TEM observation, which illustrates that there are free radicals produced.[22] Thus it can be postulated that TPT causes damage to *S. subsalsa* due to the production of large amounts of free radicals, this proposal could be further studied by electron spin resonance.

CONCLUSIONS

SEM images show a significant change in the screw-pitch of *S. subsalsa*, which suggests that TPT may damage the inheritance characteristics of *S. subsalsa*. The TEM images illustrate that in *S. subsalsa* the targets, which are easily attacked and destroyed, are the external pectin theca, limiting membrane and internal photosynthetically active lamellae in the cell. Reversible and irreversible cell damage (cell necrosis) are also observed. The mechanism for toxic the effects of TBT on *S. subsalsa* might be damage caused by free radicals. TPT was degraded to less toxic DPT and trace amounts of MPT by *S. subsalsa*.

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