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Electron microscopy of the marine microalga *Dunaliella tertiolecta* exposed to triphenyltin

HM Mooney and JW Patching

The Martin Ryan Marine Science Institute, National University of Ireland, Galway, Ireland

Chemostat-grown cells of the chlorophyte *Dunaliella tertiolecta* (Butcher) exposed to triphenyltin were examined using transmission electron microscopy. Following a 1-h exposure to 21 and 84 μM triphenyltin, mitochondria underwent structural damage and the thylakoid membranes of a small proportion of cells spread from the usual compact arrangement. Prolonging the exposure time resulted in significant cell lysis in cultures exposed to 84 μM triphenyltin.

Keywords: phytoplankton; organotin; triphenyltin; transmission electron microscopy; chlorophyte; *Dunaliella tertiolecta*

Introduction

Organotins have widespread applications in industry and agriculture. Their specific uses as biocides, industrial catalysts and polymerizing agents, often dictate their fate as pollutants in the aquatic environment. In the early 1980s, it became clear that trisubstituted organotins used in antifouling paints were toxic to a wide variety of non-target marine organisms, in addition to the fouling species found on submerged surfaces. As a result, limitations were placed on the use of organotins as active agents in antifouling paints. Legislation restricted the use of both organotins in general (Ireland, France), and butyltins in particular (UK, USA, Japan). Restrictions on the use of triphenyltin (TPhT) are not as widespread as the legislative banning of tributyltin. TPhT is used in agriculture as a fungicide (commercial names Brestan [18], Du-Ter [3]) and also as the active ingredient in antifouling paints, either as a co-polymer with rubber, or mixed with copper or tributyltin [9].

Surveys of aquatic habitats have revealed levels of phenyltins which could pose a threat to non-target organisms [1,2,8,25,28]. Concentrations range from hundreds of nanograms per liter in harbor water [1] to thousands of nanograms per kilogram of shellfish in contaminated lakes and harbors [8]. Triphenyltin is toxic to a variety of marine organisms including rainbow trout fry [7], marine yeasts [15] and phytoplankton [2,16,17,24,27,31,32].

Exposure of phytoplankton to pollutants may have wide-reaching effects in aquatic ecosystems, either by initiating a chain of bioaccumulation [12,14,22] or by inhibiting the flow of energy into food webs. Organotins including TphT inhibit microalgal growth [5,16,24,27,31] as well as disrupting biochemical processes such as photosynthesis [4,17] and respiration [17]. Transmission electron microscopy has shown changes in the fine structure of algal cells after exposure to organic toxicants (such as chlorinated benzenes and pesticides), and heavy metals [29,33]. Both organic and inorganic toxicants are capable of altering the fine structure of algal cells, with no obvious correlation between damage by either type of pollutant [19]. Despite these studies, more needs to be determined about the mechanism of toxicity which, in extreme cases, can lead to total breakdown of membranous organelles in algal cells.

Short-term studies on the effects of tri- and diphenyltin on continuous cultures of the marine microalga *Dunaliella tertiolecta* revealed that micromolar concentrations of these compounds inhibited respiration and photosynthesis [17]. This paper reports the effects of similar exposures to TPhT on the cytoplasmic fine structure of the organism.

Materials and methods

Triphenyltin chloride (TPhT) was obtained from Pfaltz and Bauer (Stamford, CT, USA). Stock solutions were prepared by dissolving the organotin in absolute ethanol such that a transfer of 500 μl to a liter of culture yielded the required concentration.

A unialgal culture of *Dunaliella tertiolecta* (Butcher) CCAP 19/27, was obtained from the Culture Collection of Algae and Protozoa, Dunstaffnage, Scotland. A continuous culture was grown under constant illumination in f/2 medium [10] at 17°C, and a dilution rate of 0.0096 h^{-1} as described in Mooney and Patching [17].

For exposure to TPhT, 500 ml of continuous culture was divided equally into six sterile, glass 250-ml conical flasks. To tests were added 42 μl TPhT stock solution while controls received the same volume of ethanol. Cell suspensions were illuminated and incubated at 17°C for the duration of exposure to phenyltin.

Cells were collected by gentle centrifugation at 3600 × *g*, and fixed overnight in 3% gluteraldehyde in 0.2 M sodium cacodylate buffer at pH 7.2. Cells were postfixed for 1 h with 2% osmium tetroxide in cacodylate buffer. The fixed material was washed once in cacodylate buffer prior to dehydration. All fixatives and the cacodylate buffer were prepared in membrane-filtered (Nucleopore, 0.22-μm pore size) sea water. Samples were dehydrated in a series of 10, 30, 50, 70, 90 and 100% (vol/vol of distilled H_2O) acetone for 20 min each. Dehydrated cells were suspended in a

Correspondence: JW Patching, Marine Microbiology Section, The Martin Ryan Marine Science Institute, National University of Ireland, Galway, Ireland
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50 : 50 mixture of resin and acetone for 1 h and then embedded in 100% Spurr's resin. Embedded samples were polymerized at 60°C for 3 days and sectioned using an ultramicrotome. Thin (60–90 μm) sections were picked up on 200-mesh copper grids and post-stained with uranyl acetate for 30 min at 40°C. After rinsing in distilled water, sections were post-stained with lead-citrate for 5 min at room temperature, and finally rinsed in distilled water. Sections were viewed under EM (Hitachi-700) at an accelerating voltage of 75 kV. A total of 50 random cells from a minimum of three grids containing thin sections were examined, and structural abnormalities observed in cells were expressed as a percentage of this.

Results

Cells of *D. tertiolecta* (Figure 1) from TPhT-free control incubations were ellipsoidal and approximately 8 μm long and 4 μm wide. In cross-section a cup-shaped chloroplast was visible. The thylakoid membranes of the chloroplast ran parallel to each other. In most cases, inter-thylakoidal spaces housed osmophilic granules which appeared as small, round electron dense spots. The thylakoid membranes of the chloroplast protruded into a single pyrenoid which was also tightly bounded by starch plates. The nucleus was usually found at the center of the cytoplasm near one of the cell poles. Also present were one or more mitochondria, usually oblong or spherical in shape when viewed in cross-section (Figure 1b). Cells contained a golgi body and one or more contractile vacuoles. Figure 1a is representative of over 70% of control cells while the remaining 30% appeared to be dead or dying on the basis of their irregular shape and poorly defined organelles, and the incidence of cell lysis. The appearance of the healthy cells matches the ultrastructural description of this organism given by Hoshaw [11], who noted also that the thylakoids were surrounded by an outer membrane and that the nucleus was bound by a nuclear envelope.

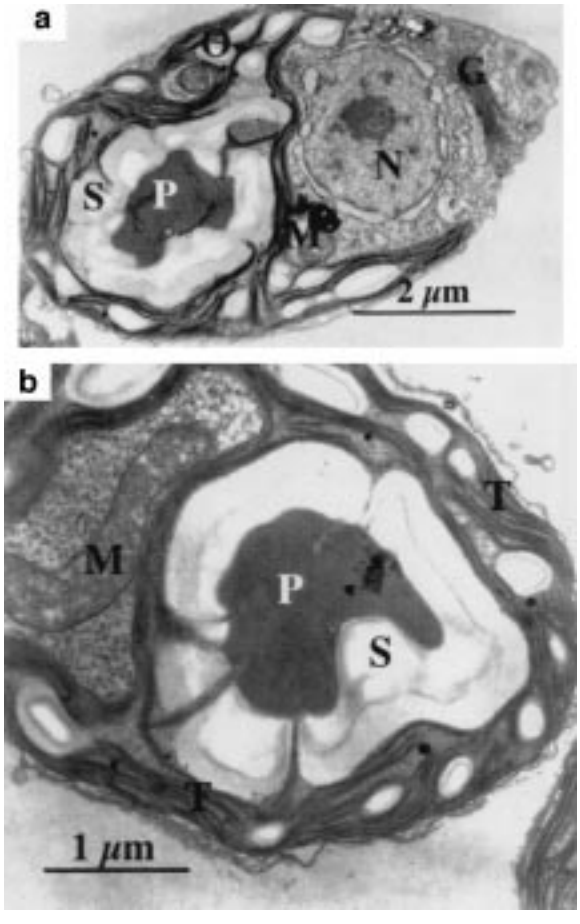

Figure 1 *D. tertiolecta* unexposed to phenyltin. (a) General view of cell; (b) detail of cell showing mitochondrion (M). T, thylakoid membranes; S, starch; P, pyrenoid; N, nucleus; O, osmophilic granules; G, golgi body.

Cell ultrastructure was visibly affected by exposure to 21 μM TPhT for 1 h (Figure 2). The mitochondria in over 45% of cells, which otherwise structurally resembled healthy control cells, were expanded (Figure 2a). A small proportion of cells ($<$ 5% of intact cells: Figure 2b) contained thylakoid membranes which appeared to have spread from the compact arrangement of lamellae seen in control cells and osmophilic granules (Figure 2b) which were larger than those in control cells. Approximately 20% of the intact cells examined contained a pyrenoid which was partially exposed rather than completely surrounded by starch (Figure 2c). Increasing the exposure time to 8 h did not result in a greater extent of mitochondrial damage (Figure 3a), nor did the percentage of cells containing a partially exposed pyrenoid increase. A small number of intact cells (Figure 3b) contained thylakoid membranes in which the stacks of lamellae had spread from the usual compact arrangement seen in controls.

After 1 h exposure to 84 μM TPhT, over 60% of intact cells examined contained expanded mitochondria (Figure 4a). One to two percent of intact cells contained thylakoid membranes which appeared to have spread from the usual compact arrangement of lamellae seen in controls (Figure 4b), an exposed pyrenoid and large osmophilic granules identical to those seen in Figure 2b. These cells, however, accounted for less than 5% of the total number of cells examined. An increase in the exposure time to 8 h resulted in the lysis of over 65% of cells while those that were still intact appeared very similar to cells exposed to 84 μM TPhT for 1 h.

Discussion

This study describes changes in the cell ultrastructure of the chlorophyte, *D. tertiolecta*, induced by exposure to TPhT under conditions similar to those known to inhibit photosynthesis and respiration [17]. The most obvious effect of TPhT exposure was swollen mitochondria (Figures 2a, 3a, and 4). The structural change in mitochondria correlated with the acute effects of TPhT on respiration [17]. A secondary effect induced by TPhT was occasional disruption of the thylakoid membranes of the chloroplast (Figures 2b, 3b and 4b). The thylakoid membranes of this species envelop and protrude into the pyrenoid and starch plates and extend into other parts of the cell. The disruption involved separation of the thylakoid membranes, causing them to spread from their normal position. Similar effects have been reported in phytoplankton exposed to heavy metals and organic pollutants [13,19–21,23,26].

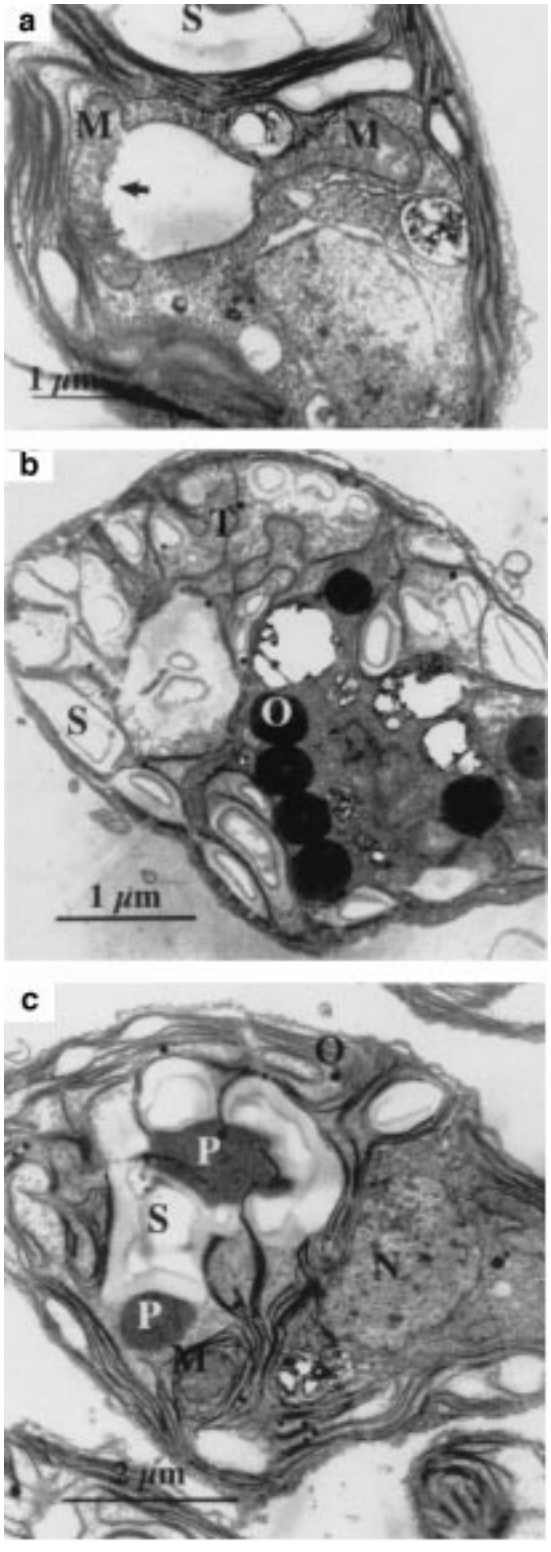

Figure 2 *D. tertiolecta* exposed to 21 μM phenyltin for 1 h. (a) Detail of cell showing expanded mitochondrion (arrow: M); (b) detail of cell showing accumulation of large osmophilic granules (O); (c) detail of cell showing exposed pyrenoid (P). T, thylakoid membranes; S, starch; O, osmophilic granules.

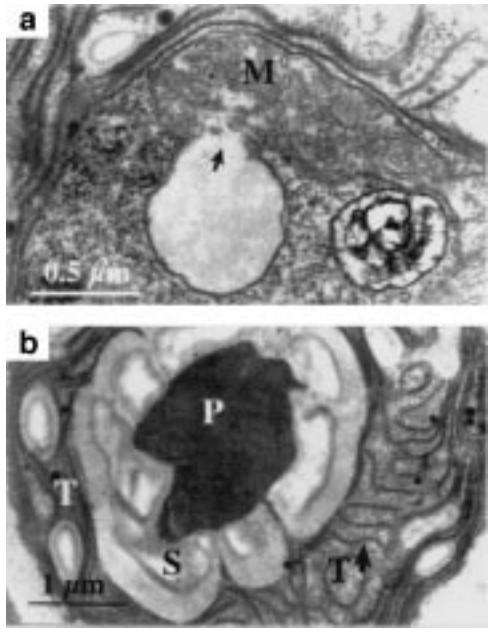

Figure 3 *D. tertiolecta* exposed to 21 μM phenyltin for 8 h. (a) Detail of cell showing expanded mitochondrion (arrow: M); (b) detail of cell showing thylakoid membranes which have spread from their usual compact arrangement of lamellae (T). S, starch; P, pyrenoid.

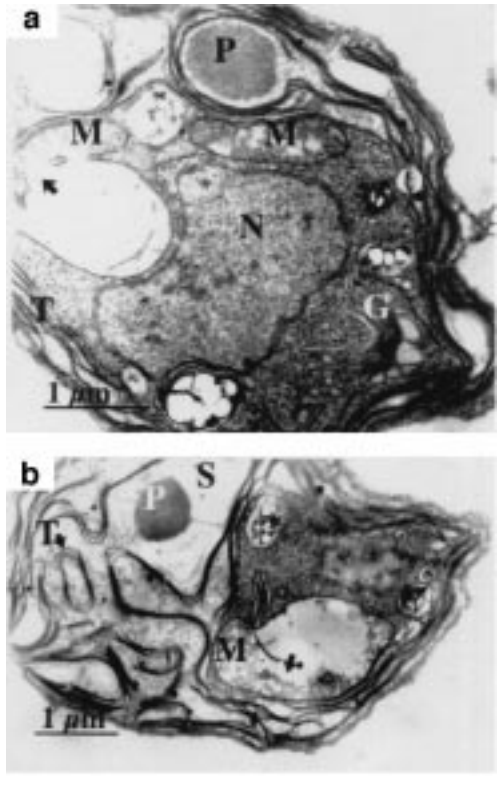

Figure 4 *D. tertiolecta* exposed to 84 μM phenyltin for 1 h. (a) Detail of cell showing expanded mitochondrion (arrow: M); (b) detail of cell showing an expanded mitochondrion (arrow: M) and thylakoid membranes which have spread from their usual compact arrangement of lamellae (arrow: T). S, starch; P, pyrenoid; N, nucleus; O, osmophilic granules; G, golgi body.

Visviki and Rachlin [30] postulate that the effect of heavy metals on algal photosynthesis involves decoupling of the photosynthetic reactions and a subsequent reduction in the size of the chloroplast [29]. Since our exposure times were low, it is possible that only the first stage of damage had taken place. Rachlin *et al* [19] have also suggested that the larger surface area of the thylakoids after heavy metal exposure may be the result of uncoupling of the protein bonds causing the lamellae to stretch. Since organotins are thought to act as uncoupling agents [6], this may explain the short-term structural effects of TPhT exposure observed in this study.

Comparison of ultrastructural changes in *D. tertiolecta* caused by sublethal concentrations of TPhT, with their effects on rates of respiration and photosynthesis [17] reveal that inhibition of these metabolic processes could take place before structural damage to the responsible organelles was observed. In this study, exposure to 84 μM TPhT for 8 h caused over 65% cell lysis, while a concentration ten times lower (results not shown) had no visible effect on cell ultrastructure. The former concentration approximates the EC_{50} value for inhibition of photosynthesis and respiration in this organism, and concentrations ten times lower were also inhibitory [17]. It could be argued, therefore, that metabolic measurements are a more accurate toxicity-testing tool.

It is significant that organelles of *D. tertiolecta*, a species which has proven relatively tolerant to organotins in previous studies involving growth measurements, have been damaged by short-term exposure to micromolar concentrations of TPhT. There are limited data on the effects of long-term exposure to organotins on phytoplankton communities. TPhT concentrations in aquatic environments are typically lower than those used in our studies [17] but longer exposure times could result in similar sublethal structural and physiological effects, with consequent changes in community structure and activity.

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