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Effects of Tin(IV) Chloride and of Organotin Compounds on Aquatic Micro-organisms

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The growth response of the alga *Chlorella kessleri* and the euglenoid *Euglena gracilis* has been studied as a model system to determine the effects of a tin salt ($SnCl_4 \cdot 5H_2O$) and of some organotin (OT) derivatives, namely tetrabutyltin (TeBT), tributyltin (TBT) and tributyltin oxide (TBTO). Abiotic degradation was studied as well. Cells were exposed to a toxicity series (0–50 $\mu g/mL^{-1}$) for the four chemicals in seven-day bioassays. Both micro-organisms are tolerant of the inorganic salt, but growth inhibition was significant for all OT compounds, and especially large for TBT and TBTO. Although *C. kessleri* and *E. gracilis* are known to be tolerant towards metals and organic chemicals, the present results show that both are sensitive to organotin compounds: the inhibition of the growth was greater for *C. kessleri*. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

Organotin (OT) compounds are used extensively as stabilizers for PVC, industrial catalysts, antifouling agents, pesticides and food preservatives, and for the manufacture of other chemicals.[1–3] OT derivatives may be introduced into food through the use of such compounds as biocides and through migration from PVC materials.[4] The World Health Organization (WHO) reported that the estimated mean total daily intake of tin by man ranges from 200 μg to 17 mg.[5, 6] Known environmental risks of OT species are caused by their high neurotoxicity even at low concentrations. Concern over the severity of these effects has led to restrictions on the use of OT compounds in some Western countries,[7] but, in spite of those restrictions, the worldwide annual production of OT compounds is increasing year by year. The presence of organotin compounds and of their degradation products in sea[8–12] in fresh-water bodies[13] and in sediments[14–16] has recently attracted much attention also, because of their detrimental effects on aquatic organisms, especially bivalve and gasteropods molluscs,[17] fish and birds.[18]

In particular, tributyltin (TBT) compounds are present in antifouling paints as active ingredients and continue to pose a major ecotoxicological threat;[19] apart from imposex and growth-related effects of TBT on molluscs, there are other concerns such as contamination of fish products resulting in contamination of human food.[20, 21] As a result of the uptake of these compounds into the human body, TBT has been determined in biological samples,[22, 23] including human hair.[1] Dibutyltins (DBT) are reported to be teratogenic in rats; moreover severe maternal toxicity occurred after treatment with butyltin and TBT.[4] On the other hand, the neuropathological sequelae in rats exposed to trimethyltin (TMT) have recently been well documented.[24] It has been also reported that acute exposure to TMT produces a behavioural syndrome in adult rats which includes convulsive episodes and hyperactivity combined with learning deficiencies.[25] Although tetrabutyltin (TeBT) does not have any large-

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scale commercial outlets at present, it is an important intermediate in the manufacture of other OT derivatives, R_nSnX_{4-n}, from $SnCl_4$ and other chemicals.[3]

Uptake of OT species has been studied in detail, mostly for marine organisms and also for some freshwater organisms, but most of them do not include all the components of the foodweb. Modelling and risk assessment of TBT accumulation in the food web of a shallow freshwater lake have been recently reported.[27] The present work aims to gain insight into the effects of OT species on the lowest levels of the ecosystem. *Euglena gracilis* was chosen as test species on the basis of its known tolerance of many organic toxicants, including a wide range of antibiotics, as well as of heavy-metal pollution.[28] The response of *Chlorella kessleri* (a green alga) was also assessed. Of the OT species most widely used, tributyl and triphenyl forms appeared to be more toxic to freshwater and marine microalgae.[29] Although biotic degradation was determined to be primarily responsible for the degradation of the OT compounds, the present work also attempts to gain some insight into the kinetics of the abiotic degradation.

MATERIALS AND METHODS

Reagents

Hexane was refluxed for several days on concentrated sulphuric acid that was renewed daily; the hexane was then distilled, treated with sodium hydroxide and distilled again from disodium benzophenone dianion. n-Butyl-lithium was prepared by a modified version of the procedure previously described.[30, 31] Lithium wire (1.35 g, 193 mmol) was cut into small pieces and placed in a flask containing boiling hexane (80 ml), then the flask was capped with an air-tight stopper and kept at 50 °C. Butyl chloride (10 ml, 96 mmol) was syringed in small aliquots into the flask during 3 h and the mixture left to react for 1 h at 50 °C. The butyl-lithium concentration was determined by the double-titration method and/or by the reaction with diphenylacetic acid as previously described.[32]

Commercial tributyltin oxide (TBTO) was used without further purification. Hydrated tin(IV) chloride, $SnCl_4 \cdot 5H_2O$, was made anhydrous as follows: 60 g (0.17 mol) of $SnCl_4 \cdot 5H_2$ was refluxed with thionyl chloride (90 ml) until the evolution of gases had ceased.[33] The reflux was continued for an additional hour, then the excess of thionyl chloride was distilled. This was followed by distillation of the anhydrous SnCl, which was kept under a nitrogen atmosphere and distilled again under nitrogen immediately before use. Tetrabutyltin (TeBT) was prepared by a modified version of the method described for tetraphenyltin (TePhT),[34] as follows: a solution of an excess of butyl-lithium in hexane (10 mmol) was added, by a syringe, to a suspension of tin(IV) chloride (2 mmol) in hexane at 0 °C. The reaction was practically instantaneous. After the reaction mixture had been treated with water, the organic layer was concentrated and purified by TLC (85% yield). (TBT) Tributyltin chloride and (DBT) dibutyltin chloride were prepared similarly to the method described for TeBT, but using the stoichiometric amounts of butyl-lithium required in each case.

Algal cultures

The euglenoid *Euglena gracilis* was obtained as pure isolates from samples collected in the Matanza River (Buenos Aires, Argentina). The strain was growth and maintained axenically in *Euglena gracilis* medium (EGM) at a pH of 6.5 in 125-ml Erlenmeyer flasks. The flask cultures, containing 50 ml of medium, were incubated at 25 °C and a 16:8 light/dark cycle. Stock and experimental cultures were maintained in the log phase of growth by removing medium and cells every seven days and replacing them with an equal volume of fresh sterile medium.

The alga *Chlorella kessleri* was isolated in the Laboratory of Plant Physiology, Universidad de Buenos Aires, and maintained in a modified Bold basal medium (BBM) with glucose under the same laboratory conditions.

Methods

Kinetics

A 2.0 mM solution of the organotin compounds in twice-distilled ethanol was prepared. When necessary, the pH was adjusted to 4 with HCl. Decalin or biphenyl was added as internal standard for the GLC analysis. Aliquots of 1 mL of the reaction mixture in sealed ampoules were placed at once in a thermostat at 50 °C, 60 °C and 80 °C, respectively. Samples were taken at time intervals and analysed by gas chromatog-

raphy (GC). The OT concentrations used in the present work allow direct GC determination without previous derivatization, as in the more recent studies.[20, 35]

Analytical GC was performed on a Hewlett–Packard 5890 Series II Plus instrument equipped with a flame ionization detector (FID). GC conditions were as follows: column, HP-5 (5% diphenyl- and 95% dimethyl-polysiloxane); injection port temperature, 320 °C; detector temperature, 300 °C; oven temperature program, 50 °C held for 2 min, increased at 15 °C min^{-1} to 240 °C, held for 10 min. Under these conditions the alkyltins can be reliably determined. Identification of the compounds was carried out by comparison of their retention times against standards and by GC–MS using an HP-5890 gas chromatograph coupled to a BG Trio-2 mass spectrometer.

Algal assays

Test solutions consisted of each medium as control or solutions containing the toxicants TeBT, TBT and $SnCl_4$ at the concentrations 10, 30 and 50 μg ml^{-1}, and TBT and TBTO at 2, 5 and 10 μg ml^{-1}, in each case. All control and test concentration trials were run at least in duplicate, for a maximum 144 h exposure period. Cell density was estimated by spectrophotometry ($\lambda = 680$ nm) of well-mixed cultures, every 24 h, using a Shimadzu UV-1601 PC spectrophotometer. Since the absorbance values for each flask of a duplicate run were within 10% of each other, the data for each pair of duplicates were pooled.

At the end of the experiment a growth–response curve was constructed from the absorbance values plotted against time of exposure for the organotin compounds for each concentration of the series.

Electron microscopy

At the conclusion of the algal assays, test cultures exhibiting growth inhibition were centrifuged to a pellet and transferred to small vials. The cells were fixed for 3 h with 4% glutaradehyde and buffer at 4 °C and post-fixed in 2% osmium tetroxide (same buffer) for 1 h; then they were dehydrated through a graded ethanol series. After dehydration, the cells were embedded in 100% Spurr's medium. The specimen blocks were then polymerized at 70 °C overnight. Thin sections were prepared with a diamond knife mounted on a microtome, then they were stained with uranyl acetate and post-stained with lead citrate. The fine structure of the cells was examined by means of transmission electron microscopy (TEM) using a Siemens Elmiskop 1 microscope.

RESULTS AND DISCUSSION

Abiotic degradation

Prior to studying the biotransformation of OT species, the likelihood of degradation in the absence of micro-organisms under similar conditions to those in the bioconversion experiments was examined. Since almost no degradation was observed in aqueous media at room temperature, accelerated ageing was carried out in aqueous ethanol at 50, 60 and/or 80 °C, to assess abiotic degradation; results could then be extrapolated to room temperature by previously reported procedures.[36, 37] In some cases, degradation was also studied under more drastic conditions to assess the determination levels. Critical considerations with respect to the identification of tin species in the environment have been recently reported.[38]

Tetrabutyltin

Table 1 shows the results of the study of TeBT in 96% ethanol at 60 °C for 88 days. It can be observed that no reaction occurs during that time; the percentage recovery shows the reliability of the quantitative assay method, the error being not more than ±10%. The same study, carried out at 80 °C, showed that a new compound (X) appeared after 20 days. The compound X was characterized by its GC retention time and mass spectra as 1,4-diethoxybutane. From these studies, it can be concluded that the TeBT undergoes very slow degradation in aqueous ethanolic solution. Its permanence in the environment can be grossly estimated as >2 years and it certainly does not undergo abiotic degradation during the time the bioconversion experiments were carried out.

Tributyltin

The abiotic degradation of TBT was studied at neutral pH and also in the presence of HCl (pH 4) to examine the effect of low-pH media. The reaction was extremely slow at room temperature, but it could be studied by accelerated ageing in aqueous ethanol at 50, 60 and

Table 1. Degradation of TeBT in 96% ethanol at pH 7

$T = 60$ °C			$T = 80$ °C			
Time (days)	10^2 [TeBT][a] (M)	Recovery of TEBT (%)	Time (days)	10^2 [TeBT][a] (M)	Recovery of TeBT (%)	10^2[X] (M)
7	0.164	89	3	0.160	86	0.000
12	0.166	90	8	0.160	86	0.000
19	0.159	86	13	0.151	82	0.000
24	0.157	85	17	0.147	79	0.000
29	0.145	78	25	0.135	73	0.070
45	0.162	88	30	0.147	79	0.230
52	0.190	103	34	0.134	72	0.270
61	0.177	96				
73	0.182	98				
88	0.180	97				

[a] $[TeBT]_0 = 0.00185$ M.

80 °C. Table 2 shows the results of the kinetics at 60 °C at both pH values for 38 days. It can be observed that the reaction at pH 4 is *slower* than at pH 7, in spite of what can be expected for an acid-catalysed degradation. Similar results were observed at 80 °C; as can be observed in Fig. 1, the reaction follows pseudo-first-order kinetics, and the calculated reaction rate coefficient for the reaction at pH 7 is $k = (6.3 \pm 1.2) \times 10^{-8}$ s^{-1} ($t_{1/2} = 4.5$ months). At both temperatures, the formation of 1,4-diethoxybutane at neutral pH shows a fast increase in rate after a short induction period, and then stabilizes: the best kinetic treatment is that for an autocalysed reaction and the estimated reaction rate coefficient was 1.47×10^{-3} M^{-1} s^{-1}. No further study of this reaction was considered relevant for the purpose of this work. On the other hand, no DBT or monobutyltin (MBT) was detected in the abiotically degraded samples.

Algal assays

Table 3 shows the rate of growth of *E. gracilis* and *C. kessleri* in the presence of 10, 30 and 50 μg ml^{-1} of tin(IV) chloride. It can be observed that the growth of *E. gracilis* is not affected by inorganic tin since the absorbances are similar to those measured in the absence of the inorganic salt. This is consistent with the known tolerance of *E. gracilis* to metals. *C. gracilis* exhibits a faster growth in the same period of time and it is also tolerant to the presence of $\leqslant 50$ μg ml^{-1} of tin(IV) chloride. The results shown in Table 3 are useful for estimating the reliability of the spectrophoto-

Table 2. Degradation of TBT in 96% ethanol at $T = 60$ °C

	pH = 7			pH = 4		
Time (days)	10^2 [TBT][a] (M)	Recovery of TBT (%)	10^2[X] (M)	10^2 [TBT][b] (M)	Recovery of TBT (%)	10^2[X] (M)
4	0.160	100	0.000	0.147	86	0.000
6	0.161	101	0.004	0.134	78	0.004
7	0.121	76	0.005	0.127	74	0.004
10	0.114	71	0.012	0.132	77	0.005
12	0.112	70	0.021	0.121	71	0.007
14	0.143	89	0.041	0.155	91	0.007
17	0.140	87	0.089	0.150	88	0.009
27	0.121	76	0.128	0.154	90	0.018
38	0.103	64	0.142	0.100	58	0.034

[a] $[TBT]_0 = 0.00160$ M. [b] $[TBT]_0 = 0.00171$ M, $[HCl]_0 = 0.0001$ M.

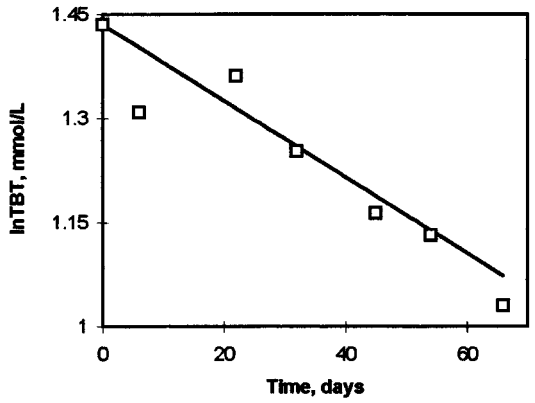

Figure 1 Abiotic degradation of tributyltin chloride, TBT, in 96% ethanol at 80 °C (pH 7).

metric method developed in this work to follow the rate of growth.

A similar experiment, but with the metal in the form of TeBT and TBT, was carried out and the results for *E. gracilis* are compiled in Table 4. It can be observed that in the presence of TeBT the rate of growth shows a marked decrease which is proportional to the concentration of the organotin compound (see Fig. 2). Similar results were observed with *C. kessleri*, which are shown in Table 5: the decrease in growth is proportional to [TeBT]. Tetraorganotins, R_4Sn, have been reported to exhibit a delayed toxic action, which may be due to their conversion to a triorganotin compound, R_3SnX, in the organisms.[26]

Figure 3 shows the response of *E. gracilis* to TBT, using the same concentration as that in the study with TeBT. It can be observed that TBT is highly toxic for *E. gracilis*; a steep decrease in the measured absorbances is observed for the three concentrations used. Since as early as at 24 h of exposure, growth was highly inhibited (see Fig. 3), the assay was finished after 96 h. *C. kessleri* has a similar sensitivity to the presence of TBT at [TBT] > 10 $\mu g\ ml^{-1}$, as shown in Table 5. Taking into account these results new experiments were carried out using lower amounts of TBT and lower cell densities in order to be able to follow the assay for longer periods of time. The assays of Fig. 3 were also compared at [TBT] = 10 $\mu g\ ml^{-1}$. The results for *E. gracilis* are shown in Fig. 4. It can be observed that even doses as low as 2 $\mu g\ ml^{-1}$ are highly toxic and the inhibition of growth is almost complete after 24 h of exposure. It is noteworthy that the concentrations used in the present work are similar or lower than in previously reported studies using other micro-organisms.[11, 12, 16] For the case of *Saccharomyces cereviasiae*, which is known to induce methylation, previously reported experiments have used concentrations of various tin species as high as 2 $g\ l^{-1}$.[39]

A recent study on the effect of TBT on the marine diatom *Phaeodactylum tricornutum* showed that algal growth was much reduced by concentrations of 2.0–20.0 $\mu g\ l^{-1}$, with reduction of cell density and cell death occurring within a few days at higher TBT concentrations,[40] similar results were observed for

Table 3. Growth of *E. gracilis* and *C. kessleri* in the presence of tin(IV) chloride[a]

		[Tin (IV) chloride ($\mu g\ ml^{-1}$)]			
Alga	Time (days)	0	10	30	50
E. gracilis	0	0.306	0.306	0.306	0.306
	1	0.351	0.350	0.338	0.341
	2	0.398	0.412	0.398	0.399
	3	0.409	0.416	0.424	0.422
	5	0.552	0.558	0.556	0.585
	6	0.597	0.608	0.628	0.657
C. kessleri	0	0.266	0.266	0.266	0.266
	1	0.520	0.412	0.478	0.369
	2	0.566	0.611	0.626	0.453
	3	0.696	0.685	0.704	0.695
	5	0.790	0.813	0.808	0.786
	6	0.839	0.852	0.822	0.794

[a] Growth was followed spectrophotometrically by measuring the absorbant at $\lambda = 680$ nm different time intervals.

Table 4. Growth of *E. gracilis* in the presence of TBT and TeBT[a]

		[Organotin] (μg ml^{-1})			
Tin species	Time (days)	0	10	30	50
TeBT	0	0.375	0.375	0.375	0.375
	1	0.425	0.400	0.384	0.352
	2	0.471	0.446	0.414	0.368
	3	0.568	0.472	0.434	0.379
	4	1.082	0.514	0.442	0.398
TBT	0	0.375	0.375	0.375	0.375
	1	0.425	0.291	0.263	0.266
	2	0.471	0.273	0.231	0.242
	3	0.568	0.263	0.230	0.241
	4	1.082	0.237	0.215	0.206

[a]Growth was followed spectrophotometrically at $\lambda = 680$ nm (see Table 3).

triphenyltin (TPT).[35] On the other hand, inhibitory effects on the growth of the marine microalga *Pavlova lutheri* were recently reported for [TPT] as low as 23 nmol l^{-1}.[41]

At the end of the present bioassays, the cells exposed to the highest concentration of TBT and TeBT (50 μg ml^{-1}) were prepared for TEM to examine the structural changes in response to contaminants. The cultures treated with TeBT showed a variable degree of cell deterioration, whereas the damage due to TBT was such that no organelles could be distinguished.[42]

Finally, a brief study was carried out with TBTO, another OT used as a biocide. Table 6 shows the results for the effects of [TBT] and [TBTO] in the range 2–10 μg ml^{-1} on the

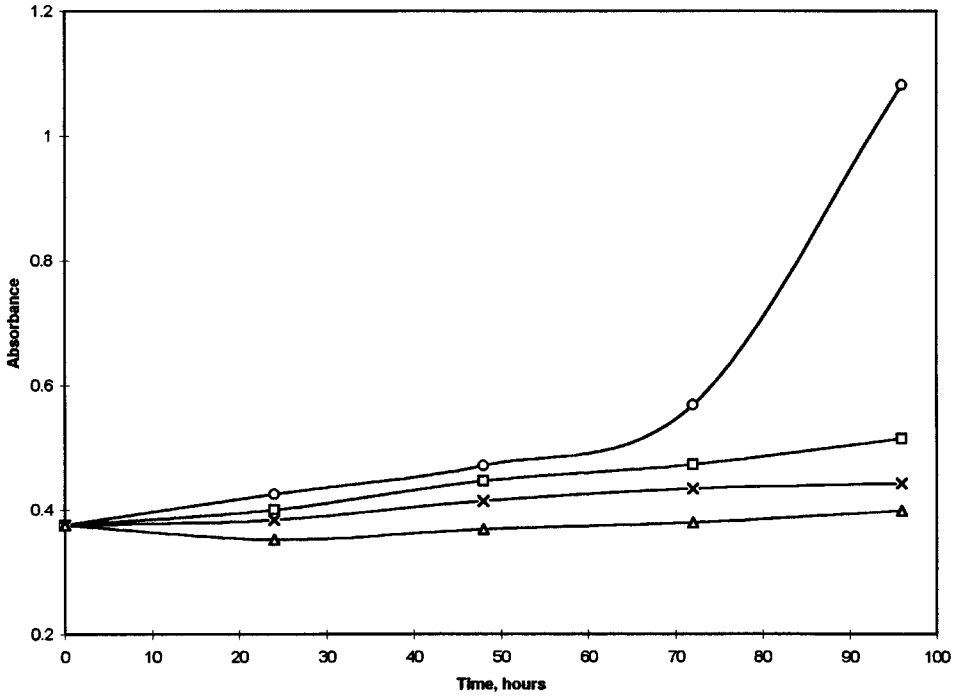

Figure 2 Growth of *E. gracilis* in the presence of tetrabutyltin, TeBT, followed by measuring the absorbance ($\lambda = 680$ nm) at different time intervals. [TeBT]: ○, 0 μg ml^{-1}; □, 10 μg ml^{-1}; ×30 μg ml^{-1}; △, 50 μg ml^{-1}.

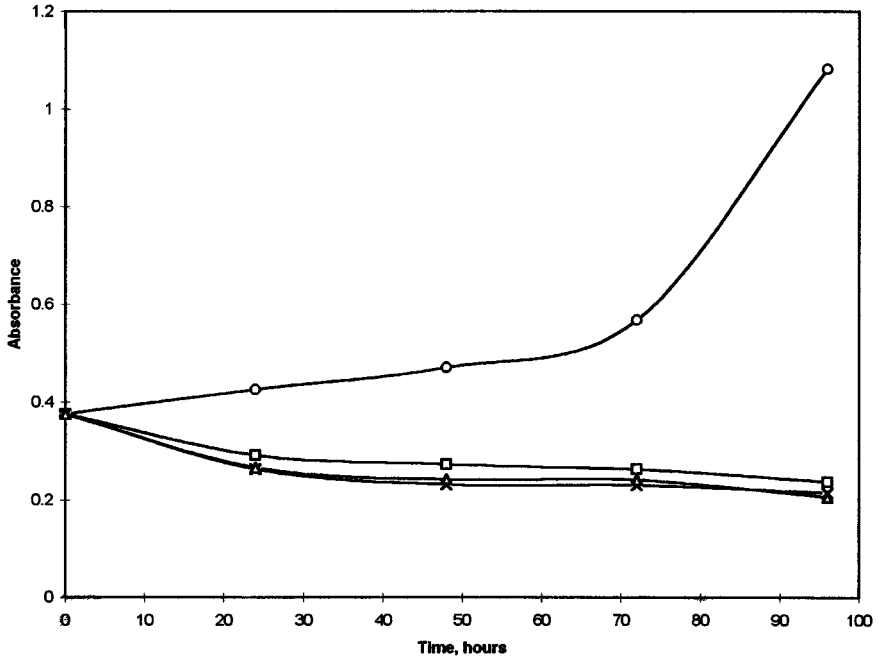

Figure 3 Growth of *E. gracilis* in the presence of tributyltin chloride, TBT, followed by measuring the absorbance ($\lambda = 680$ nm) at different time intervals. [TBT]: ○, 0 μg ml^{-1}; □, 10 μg ml^{-1}; ×30 μg ml^{-1}; △, 50 μg ml^{-1}.

growth of *E. gracilis*. By comparison of the data it can be concluded that the response of *E. gracilis* to both tributyltin derivatives is very similar, and concentrations of TBTO $\geqslant 2$ μg ml^{-1} are highly toxic for this micro-organism. In previously reported bioconversion studies of TBTO with micro-organisms isolated from sediments, initial concentrations of 1 mg l^{-1} were tolerated.[16]

Conclusions

Although most of the studies on the effects of OT compounds has been carried out on marine

Table 5. Growth of *C. kessleri* in the presence of TBT and TeBT[a]

		[Organotin] (μg ml^{-1})			
Tin species	Time (days)	0	10	30	50
TeBT	0	0.266	0.266	0.266	0.266
	1	0.511	0.354	0.341	0.327
	2	0.647	0.341	0.353	0.310
	3	0.858	0.339	0.312	0.297
	5	1.068	0.411	0.306	0.295
	6	1.203	0.463	0.355	0.297
TBT	0	0.266	0.266	0.266	0.266
	1	0.511	0.300	0.268	0.304
	2	0.647	0.302	0.303	0.285
	3	0.858	0.289	0.298	0.298
	5	1.068	0.290	0.317	0.295
	6	1.203	0.357	0.339	0.306

[a] Growth was followed spectrophotometrically at $\lambda = 680$ nm (see Table 3).

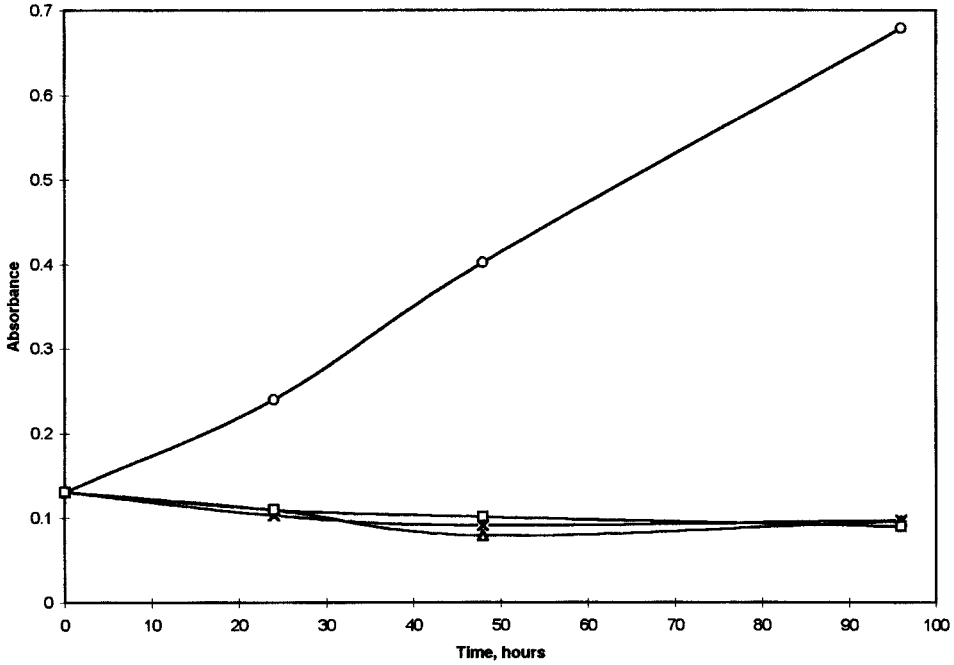

Figure 4 Growth of *E. gracilis* in the presence of tributyltin chloride, TBT, followed by measuring the absorbance ($\lambda = 680$ nm) at different time intervals. [TBT]: ○, 0 μg ml^{-1}; □, 2 μg ml^{-1}; ×5 μg ml^{-1}; △, 10 μg ml^{-1}.

organisms, the present results show that *E. gracilis* and *C. kessleri* are sensitive to [OT] $\geqslant 2$ μg ml^{-1} in freshwater environments. In all cases, the inhibition of growth was greater for *C. kessleri*. The effect of TeBT was smaller than that of TBT or TBTO. TBT and TBTO were extremely toxic for both species, even at the lowest concentrations and from the start of the experiment, whereas the response to TeBT seemed to be dependent on the concentration, and the culture would potentially recover. Studies to determine the cell damage are in progress. The present results suggest that *E. gracilis* and *C. kessleri* could be used as indicators for OT contamination in aquatic environments.

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Table 6. Growth of *E. gracilis* in presence of TBT and TBTO[a]

		[Organotin (μg ml^{-1})			
Tin species	Time (days)	0	2	5	10
TBT	0	0.131	0.131	0.131	0.131
	1	0.240	0.103	0.109	0.110
	2	0.402	0.091	0.079	0.101
	4	0.679	0.097	0.096	0.090
TBTO	0	0.131	0.131	0.131	0.131
	1	0.240	0.113	0.099	0.117
	2	0.402	0.117	0.098	0.105
	4	0.679	0.120	0.094	0.096

[a] Growth was followed spectrophotometrically at $\lambda = 680$ nm (see Table 3).

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