Journal of Industrial Microbiology, 2 (1988) 373–378
Elsevier

SIM 00102

Toxicity of methyltins to microbial populations in estuarine sediments

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Received 16 April 1987
Revised 12 October 1987
Accepted 15 October 1987

Key words: Organotin; Methyltin; Tin; Estuarine microorganism

SUMMARY

The toxicities of three organotin compounds were examined on natural populations of microorganisms in sediments from Boston Harbor. Mono-, di- and trimethyltins were toxic to organisms from these sediments, and the di- and trimethyl compounds were more toxic than the monomethyl compound as measured by either viable counts or by [^{3}H]thymidine uptake. Approximately three to eight times as much organotin was required to achieve the same effect measured by thymidine uptake as measured by viable counts. The results of replica plating experiments suggest that most estuarine organisms which are resistant to one methyltin will be resistant to other methyltins. LC-values suggest that at concentrations reported for methyltins in aquatic environments, methyltins alone are not likely to cause major alterations in the microbial flora. However, these compounds may combine with other stressors to alter the composition of natural populations.

In 1976, about 200×10^6 kg of tin was used in the world, of which 28×10^6 kg (14%) was used in the form of organotins. The annual growth in organotin consumption during the period 1978–1988 was predicted at 11–13% [38]. Organotins are used as stabilizers for poly(vinyl chloride) (PVC) and for other plastics and paints, as industrial catalysts, as industrial and agricultural biocides, as wood-preservatives and antifouling agents, and for a variety of other purposes. About two-thirds is used for PVC stabilization and nearly a tenth is used for biocides [6]. Much of this organotin will eventually reach the aquatic environment.

Of particular interest in aquatic environments are tributyltin, which can leach from antifouling paints on boat hulls, and methyltins, which can be formed by chemical and microbiological processes [1,4,5,7,8,11,14]. In addition, harbor sediments, which are known to receive tributyltins as their principal source of tin, have been shown to contain methylated derivatives of butyltins [23]. This suggests that methylation may be involved in the transformation of butyltin species. Ridley et al. [28] proposed a geochemical cycle involving methyltins, but the cycle has not yet been demonstrated to occur in

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aquatic systems. Thompson et al. [31] recently reviewed organotin compounds in the aquatic environment.

Tin and organotins can inhibit or kill aquatic microorganisms [9,10,12,13,15,19,20,26,34,35], but most studies have been conducted on pure cultures. Therefore, we examined some effects of methyltin compounds on natural microbial populations. We concentrated on organisms in surficial sediments and at the sediment-water interface because much of the tin in aquatic systems is in the sediments [12,24,31 (review)].

MATERIALS AND METHODS

Sample collection. Sediment samples were collected from the intertidal zone of Savin Hill Cove in Boston Harbor, MA, U.S.A. At low tide, the top 1–2 cm of surface sediment was taken with a sterile spatula and immediately placed in a sterile Whirlpac® bag. The samples were transported to the laboratory and processed within 2 h.

Media preparation. A dilute modified estuarine nutrient medium (dMAM) was used for the enumeration of microorganisms. The medium contained: peptone, 0.1 g; yeast extract, 0.1 g; $FeCl_3$, 0.06 g; K_2HPO_4, 0.05 g; agar (Difco Laboratories, Detroit, MI), 20 g; 500 ml distilled water and 500 ml filtered and aged (more than 4 weeks) estuarine water (salinity approx. 30‰). The medium was autoclaved and then the pH was adjusted to 7.2 with sterile 1 N NaOH. To enumerate tin-resistant microorganisms, the medium was supplemented with either mono-, di- or trimethyltin chlorides, or with tetramethyltin at tin concentrations ranging from 1 to 1000 mg · liter^{-1}. Tin compounds were purchased from Alfa Products (Danvers, MA) and were not purified further. All methyltin chlorides were dissolved in sterile distilled deionized water and added to the medium aseptically after it had been autoclaved and cooled to 50–55°C. Tetramethyltin was added as the neat liquid. After the introduction of the tin compound, the medium was swirled to distribute the tin and plates were poured immediately. Plates were dried for 48 h at room temperature before being used.

Enumeration of microorganisms. Sediment was diluted 1:10 (w/v) with autoclaved aged estuarine water or with estuarine salts solution which contained: NaCl, 10.0 g; $MgSO_4 \cdot 7H_2O$, 5.72 g; KCl, 0.3 g; distilled water, 1.0 liter. The slurry was homogenized in a blender for 20 s and serial dilutions were prepared before the slurry settled. Appropriate dilutions were plated, in triplicate, on the surface of the plating medium. Plates were incubated at 20 ± 2°C for 2 weeks and colonies were then counted.

Replication. Plates with between 20 and 120 cfu were replicated using white velveteen onto agar medium containing another methyltin. A single velveteen was used to replicate no more than six plates. Plates were incubated for 2 weeks and colonies were counted.

[^{3}H]Thymidine incorporation. Sediment was diluted 1:100 (v/v) with sterile aged estuarine water and 10 ml were added to each of three flasks containing 100 ml of the desired concentration of methyltin. Each flask also received 100 μl of a solution containing 10 μCi of [^{3}H]thymidine (specific activity 80 Ci/mM). Formaldehyde was added to the contents of a fourth flask to serve as a killed control. Flasks were incubated for exactly 1 h, after which 10 ml of ice-cold 10% trichloroacetic acid (TCA) were added and the contents were filtered through a 0.2-μm (pore size) Nucleopore filter. Each filter was rinsed twice with ice-cold 5% TCA, placed in a scintillation vial and 0.2 ml of 60% $HClO_4$ and 0.4 ml of 30% H_2O_2 were added. Each vial was heated over steam for 1 h, and then cooled to room temperature. Scintillation cocktail (Aquasol, NEN Research, Boston, MA) was added to each vial and the vials were allowed to stand overnight at room temperature before counting the radioactivity.

Other methods. We attempted to assess the toxicity of methyltins to microbial activity in sediment using 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) reduction coupled with 4′,6-diamidino-2-phenylindole (DAPI) staining by several modifications of the procedure described by

Porter and Feig [27]. In addition, we used a modification of the spectophotometric INT technique described by Trevors [33].

RESULTS AND DISCUSSION

Tetramethyltin was not toxic at any amount added – as high as 1 g · liter^{-1}. We attribute this to the low solubility of tetramethyltin. The effects of the other three methyltin compounds on total viable counts are shown in Fig. 1. Mono-, di- and trimethyltins were toxic to microorganisms from surficial sediments, although it should be noted that the plating medium used was more conducive to the growth of bacteria than to yeasts or filamentous fungi. The di- and trimethyl compounds were more toxic than monomethyltin, as reported for pure cultures of estuarine microorganisms [13]. In general, for organotins with larger organomoieties (ethyl through butyl), di- and trisubstituted compounds are more toxic than monosubstituted compounds.

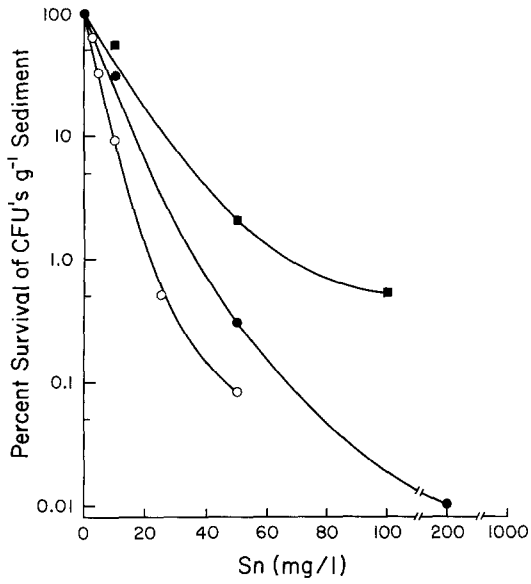

Fig. 1. Effect of methyltin compounds on total viable counts of natural populations from sediments in Boston Harbor. Tin was added as: ■, CH_3SnCl_3; ●, $(CH_3)_2SnCl_2$; ○, $(CH_3)_3SnCl$. Total viable counts in control suspensions which did not receive any addition of tin ranged from 6.6 × 10^6 to 4.7 × 10^7 · g^{-1} of sediment.

In many cases trisubstituted compounds are more toxic than disubstituted compounds against bacteria and fungi [18,31]. In most of our experiments trimethyltin was more toxic than dimethyltin, as shown in Fig. 1, but occasionally the two curves were very close or reversed.

The ability of mixed populations to take up [^{3}H]thymidine is shown in Fig. 2. The rank order of mono-, di- and trisubstituted compounds were the same as when total viable counts were used.

The relative toxicities measured by viable counts, and by thymidine uptake in a 1:100 dilution of sediment are compared in Table 1. At a given concentration of organotin, thymidine uptake was a less sensitive measure of toxicity than was colony-forming ability. Jonas et al. [17] reported that two other tin compounds, $SnCl_4$ and tributyltin chloride, were 10–100 times more toxic when measured by thymidine uptake.

Methyltins can kill estuarine microorganisms [12,13,15], including indicator bacteria [26]. Minimum inhibitory concentrations are usually in the range of 1–10 mg Sn · liter^{-1} (Figs. 1 and 2; [13,31]). Concentrations of tin and tin compounds in Boston Harbor are not known, but the highest concentrations of methyltins reported in the aquatic environment are in the range of tens to hundreds of nanograms Sn per liter [31]. Thus, it is unlikely that methyltins alone in natural water would seriously affect microbial populations in those waters, but

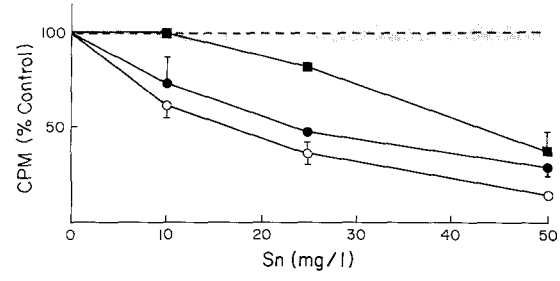

Fig. 2. Effect of methyltin compounds on uptake of [^{3}H]thymidine by natural populations of organisms from sediments in Boston Harbor. Tin was added as: ■, CH_3SnCl_3; ●, $(CH_3)_2SnCl_2$; ○, $(CH_3)_3SnCl$. The dashed line summarizes results when tin was not added and the shaded area indicates the range of the 95% confidence interval. Where error bars are not shown, the standard deviation fell within the symbol.

Table 1

Comparative toxicities of three organotin compounds to natural populations from sediments in Boston Harbor as estimated by viable counts or by thymidine uptake

Test compound	Sn concentration (mg/l)					
	viable count			thymidine uptake		
	LC_{20}[a]	LC_{50}	LC_{80}	LC_{20}	LC_{50}	LC_{80}
CH_3SnCl_3	4.3	11	17	26	43	>50
$(CH_3)_2SnCl_2$	2.9	7.2	11.6	8.0	23	>50
$(CH_3)_3SnCl$	1.5	3.7	5.9	6.2	17	45

[a] Values for LC_{20}, LC_{50} and LC_{80} are those concentrations of tin which decreased viable count or thymidine uptake by 20, 50 or 80%, respectively.

ecosystems which contain methyltin are likely to contain other stressing agents as well. Marinas, urban harbors and waste streams often contain elevated levels of other heavy metals, of toxic organic compounds, and of heavily chlorinated effluents; dissolved oxygen levels may also be low. Thus, methyltins and other organotins may contribute to cumulative stress which can alter the microbial flora of the ecosystem.

Several modifications of the microscopic and spectroscopic methods involving formazan reduction were not useful with these environmental samples, although they worked well with pure cultures of *Escherichia coli*. In the microscopic method, cells were too small to permit accurate recording of those which contained reduced formazan. In the spectroscopic method, methyltin compounds did not appear to be toxic.

Plating from environmental samples always yielded some organisms which grew in the presence of organotins, even at concentrations as high as 200 mg Sn · liter^{-1} (Fig. 1), and replica-plating experiments suggest that most organisms which are resistant to one methyltin will be resistant to other methyltins (Table 2). Lancashire and Griffiths [21] reported that strains of *Saccharomyces cereviseae* resistant to triethyltin were also resistant to trimethyltin and four other trialkyltins. It is not clear why only half the organisms isolated on medium containing monomethyl tin are resistant to dimethyltin while all of the organisms isolated on medium containing dimethyltin are resistant to monomethyltin (Table 2). This pattern was noted through five repetitions of the experiment. The situation may be similar to that with mercury resistance. In general, all bacteria which are resistant to phenyl mercuric acetate (PMA) resist $HgCl_2$ as well, but few $HgCl_2$-resistant isolates are resistant to PMA. $HgCl_2$ resistance requires one enzyme, while PMA resistance requires that enzyme and one other enzyme [29].

Isolates from natural populations are notoriously difficult to maintain in laboratory cultures, whether the maintenance medium contains tin or not. Therefore, each experiment was begun by plating dilutions from a fresh sample, and colonies

Table 2

Cross-resistance of organisms from estuarine sediment to three organotin compounds

Organisms isolated on medium containing:	Percent resistance[a] when replicated to medium containing:		
	CH_3SnCl_3 (25 mg/l)	$(CH_3)_2SnCl_2$ (10 mg/l)	$(CH_3)_3SnCl$ (10 mg/l)
CH_3SnCl_3 (25 mg Sn/l)	100	57	87
$(CH_3)_2SnCl_2$ (10 mg Sn/l)	100	100	79
$(CH_3)_3SnCl$ (10 mg Sn/l)	98	72	100

[a] In each of five experiments percent resistance was estimated using a minimum of 250 colonies and in most cases 500 colonies.

which developed as primary isolates were replicated to freshly prepared medium with no intermediate transfers.

Di- and trisubstituted organotins larger than methylated derivatives inhibit mitochondrial function in yeast [3,21,22] and disrupt cytoplasmic membrane functions in bacteria [25,30,32,36,37]. Blair et al. [2] observed that tributyltin cations bind to cells of tin-resistant estuarine bacteria. Jonas and Cooney [16] noted that estuarine organisms resistant to dimethyltin did not accumulate it, while starved but viable cells accumulated tributyltin and stannic chloride. Thus, resistance to organotins may involve passive binding in or on the cell envelope and exclusion of the toxic compound from the cell, although other mechanisms are also possible.

The present results suggest that there are one or more common mechanisms whereby microorganisms resist organotins. However, the fact that cross-resistance does not always occur suggests that methyltins can have more than one mechanism of action and that more than one mode of resistance is possible.

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