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Effects of butyltins and inorganic tin on chemotaxis of aquatic bacteria

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SUMMARY

Tributyltin (TBT) and its degradation products dibutyltin (DBT), monobutyltin (MBT) and Sn^{IV} were toxic to *Pseudomonas fluorescens* SHC-6 and *Serratia* sp. Gil-1 with EC_{50} values in the range of 10^{-3} to 10^{-4} M. These four compounds were negative chemotactic agents for *P. fluorescens*, and the butyltins were negatively chemotactic for *Serratia* sp. at concentrations over four orders of magnitude lower than the EC_{50} values. L-Aspartate was a positive chemotactic agent for both organisms. TBT, DBT and MBT negated the effect of L-aspartate on *P. fluorescens* but not on *Serratia* sp. Thus, TBT has the potential to affect microbial populations at concentrations much lower than those which prevent growth, and degradation of TBT does not always detoxify it. $SnCl_4$ was less toxic than TBT or DBT to these organisms and it was not chemotactic for *Serratia* sp. Gil-1. Tributylamine and tributylphosphate were less than 1/10th as toxic as TBT and they did not have a chemotactic effect on either organism at concentrations at which TBT had a significant effect. Therefore, both the Sn- and butyl-moieties contribute to the toxic and chemotactic properties of TBT.

INTRODUCTION

Organotin compounds are used for a variety of industrial and agricultural purposes including stabilizing plastics, preserving wood and preventing the growth of fouling organisms on surfaces immersed in water [9,42]. They are more soluble than inorganic tin compounds in lipids. Thus, they penetrate biological membranes more readily than inorganic tins and are more likely to accumulate in lipid-rich tissues or organelles. The non-polar organic moieties of organotins may become associated with the surface of biological membranes [10]. They are significantly more toxic than inorganic tins.

Much of the current interest in the toxicology and chemistry of organotins has focused on the environmental fate and effects of tributyltin (TBT), a component of many antifouling paints which have been widely employed on boat hulls in marine water and on other surfaces which are submerged in water [9,42]. TBT is toxic to some non-target organisms, particularly shellfish, at concentrations as low as 1–10 parts Sn per trillion (8.4 pM to 84 pM) [18]. TBTs can cause imposex in the common dogwhelk, which interferes with the organism's reproductive capacity [18,19]. TBT's high toxicity to nontarget organisms led to restrictions on its use in several European countries, Canada and the United States [9] but it may still be applied to vessels longer than 25 m, to aluminum-hulled boats, and to water intake pipes and other submerged surfaces.

TBT is toxic to some algae, fungi and bacteria [15,41,42]. It is important to understand the effect of organotins on microorganisms because microbes mediate a number of cyclic processes and they are at the base of food webs. In general, di- and tri-substituted compounds are more toxic than mono- or tetra-substituted compounds, and tri-substituted organotins are usually the most toxic [15]. Mixed populations of aquatic microorganisms can be killed by TBT [20,22,38,39], and so can pure cultures and natural populations of indicator organisms [38]. Toxicity can be demonstrated by loss of viability in plate counts [20,21,38], by decrease in incorporation of label from ^{3}H-thymidine [39], and by leakage of potassium from cells [14,28]. TBT's toxicity is related to its lipid-solubility. It interferes with functions of biological membranes [13]. TBT affects the same mechanisms in bacteria as in mitochondria and chloroplasts [4,5], by acting as an ionophore facilitating halide-hydroxyl exchanges or by interfering with energy transduction. TBT can be degraded both chemically and biologically in the marine water column to dibutyltin (DBT), monobutyltin (MBT) and inorganic tin. For most microorganisms, toxicity decreases with degradation: TBT > DBT > MBT > Sn^{4+}, but MBT is as toxic as TBT to some microorganisms, including bacteria [6,7], yeasts [14] and the replication of bacteriophage T_4 [16].

The toxicity of an organotin to microorganisms is usually tested by determining if it kills cells, but organotins might affect aquatic bacteria at sub-lethal concentrations. Because bacteria devote about 50 genes, some 3–5% of their genome [33], and their products to motility and chemotaxis, it is widely held that these properties provide a competitive advantage, allowing chemotactic bacteria to avoid toxic materials (negative chemotaxis) and to seek out higher concentrations of nutrients (positive chemotaxis). Chemotaxis can be easily studied [1,2,33] and motility and chemotaxis of bacteria have been the subjects of several reviews [3,12,25,30,33,36]. In addition, studies of chemotaxis might be used to understand effects of tin compounds on bacteria at the cellular and molecular level.

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The objectives of the present work were to determine if TBT and its degradation derivatives DBT, MBT and $SnCl_4$ have positive or negative chemotactic effects on selected aquatic bacteria, if they can interfere with bacterial chemotaxis toward another chemotactic attractant, and how the concentration(s) which might be involved in chemotaxis compare with concentration(s) which are lethal.

MATERIALS AND METHODS

Chemicals and media

Tributyltin chloride, $[CH_3(CH_2)_3]_3SnCl$; tributylamine, $[CH_3(CH_2)_3]_3N$; tributylphosphate, $[CH_3(CH_2)_3O]_3P(O)$; and $SnCl_4$ were obtained from the Aldrich Chemical Co., Milwaukee, WI, USA. Dibutyltin dichloride, $(C_4H_9)_2SnCl_2$, was from Alfa (Ward Hill, MA, USA) and butyltin trichloride, $C_4H_9SnCl_3$, was from Pfaltz & Bauer (Waterbury, CT, USA). For each compound an 84-mM stock solution was prepared in reagent grade methanol. Stocks were stored in the dark at 0 °C for up to 60 days, after which a fresh stock solution was prepared. Methanol was obtained from Fisher Scientific, Fair Lawn, NJ, USA.

L-Aspartate was obtained from Sigma Chemical Company (St Louis, MO, USA); a 0.5-M stock solution was prepared with sterile MilliQ water and stored in the dark at 4°C.

Washing medium contained K_2HPO_4 1.063 g, KH_2PO_4 0.531 g and NaEDTA 0.037 g per liter of MilliQ water, final pH 7.0. Chemotaxis medium was the same as washing medium but without NaEDTA. Tryptone broth contained 1% tryptone (Difco) and 0.5% NaCl per liter of distilled water, final pH 7.2. Tryptone swarm agar contained 1% tryptone, 0.5% NaCl, and 0.35% agar per liter of distilled water, final pH 7.2. Tryptone soft agar contained 1% tryptone, 0.5% NaCl, and 0.7% agar per liter of distilled water, final pH 7.2. Tryptone agar contained 1% tryptone, 0.5% NaCl, and 1.8% agar per liter of distilled water, final pH 7.2. Estuarine salts agar contained NaCl 10 g, $MgSO_4 \cdot 7H_2O$ 2.8 g, KCl 0.3 g, glucose 2 g, yeast extract (Difco) 1 g, casamino acids (Difco) 5 g, and agar 18 g per liter distilled water; the final pH was 7.2.

Organisms and culture conditions

Serratia sp. Gil-1 and *Pseudomonas fluorescens* SHC-6 were isolated in our laboratory from Boston Harbor after enrichment on medium containing TBT [44]. They were maintained on an estuarine salts agar [35] containing 10 p.p.m. (84 μM) TBT. Cultures were stored at 4°C and transferred at one-month intervals.

Cells for experiments were cultured as described by Han and Cooney [23]. Briefly, motile cells from swarm agar were sub-cultured twice in tryptone broth and log phase cells were washed and suspended gently in chemotaxis medium.

Motile cell counts were determined microscopically before and after washing by a modification of a method described by Malmcrona-Friberg et al. [32], in which the total number of cells is estimated in a suspension containing glutaraldehyde and compared to the number of non-motile cells in a suspension not treated with glutaraldehyde. Details of our use of the method are described in [23].

Estimation of EC_{50}

Cells were cultured as for chemotaxis assays. The log phase culture was diluted 10^4-fold in tryptone broth to yield a cell concentration of about 5×10^4 cells per ml; then 1.0 ml of this suspension was mixed in a test tube with 9.0 ml tryptone broth containing one of the tin compounds. After 60-min incubation at 27°C, the cultures were diluted and plated in triplicate on tryptone agar. Plates were incubated at 30°C for 24 h, and then colonies were counted and compared to counts from a control which lacked the tin compound. At least five 10-fold dilutions of each test chemical were used, and each was run in triplicate. The Moving Average Method [26] was used to estimate the concentration of each test chemical which inhibited colony formation by 50% (the EC_{50}).

Chemotaxis assay

The method is a modification of the method of Adler [2] in which the number of motile bacteria which swim into a capillary tube containing buffer and a potential chemoattractant or chemorepellent is compared with the number which swim into the control capillary tube containing buffer alone. Details of the modified method are given in [23].

After 60 min at 25°C, the lid of the chemotaxis chamber was lifted and the outside of all the capillaries was washed simultaneously by immersion in a small dish containing sterile water. The closed end was broken off each capillary and, using a small bulb supplied with the pipettes, the contents were expelled into a 1.5-ml Eppendorf tube containing 1.0 ml of tryptone broth. The contents of the tube were mixed, serial 10-fold dilutions were prepared in tryptone broth and triplicate spread plates were prepared from appropriate dilutions on the surface of tryptone agar. Plates were incubated at 30°C for 24 h and colonies were counted.

Growth during chemotaxis assays

In order to determine if the bacterial population increased during the 60-min incubation in chemotaxis medium, the washed cells were incubated in chemotaxis medium at 25°C for 60 min. At the beginning and the end of the incubation, triplicate 1.0-ml samples were suitably diluted and plated. The plates were incubated at 30°C for 24 h, and colonies were counted.

RESULTS AND DISCUSSION

Motility

Log phase cultures of *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1 contained a significant number of motile cells. The washing procedure decreased the number of motile cells (Table 1), but left a sufficient number so that each well of the chemotaxis chamber received about 5×10^6 motile cells.

Cell division and effects of solvent

Viable counts of *Pseudomonas fluorescens* SHC-6 and *Serratia* sp. Gil-1 at the beginning and end of a 60-min incubation in chemotaxis medium at 25°C were analyzed using one way analysis of variance (ANOVA). The results show that there is no significant difference at the 95% confidence interval

TABLE 1

Effect of washing on motility of *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1

Experiment number	Percentage of motile cells of:			
	P. fluorescens SHC-6		*Serratia* sp. Gil-1	
	before washing	after washing	before washing	after washing
1	55.6	28.2	26.8	11.8
2	36.8	11.9	36.6	19.7
3	43.7	25.4	29.3	20.0
Mean of total ± SEM	45.4 ± 4.5	21.8 ± 4.1	30.9 ± 2.4	17.2 ± 2.2

between the beginning and end of the incubation period. Thus, cell division during chemotaxis assay should not contribute significantly to cell numbers in the capillary tube.

Tin compounds were dissolved in methanol. The results of appropriate controls (data not shown) were analyzed by one-way ANOVA and showed that, at any concentration used in experiments, the solvent alone did not affect viability or chemotaxis during a 60-min period.

Toxicity

Table 2 shows the EC_{50} values of butyltins and related chemicals on viability of *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1. The EC_{50} concentrations of all the test compounds are greater than 10^{-5} M. Wuertz et al. [44] reported that the EC_{50} of TBT on *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1 in tryptone broth was 8.4×10^{-7} M and greater than 8.4×10^{-5} M, respectively. The EC_{50} we obtained for *Serratia* sp. Gil-1 is close to that obtained by Wuertz et al. [44], but the EC_{50} of TBT on *P. fluorescens* SHC-6 was far lower in their work than in the present work. The difference may be due to use of different media in toxicity assays, particularly since the medium can influence the apparent toxicity of tin compounds [22]. It is also possible that, in the present work, a mutant with higher TBT resistance was selected from swarm agar plates.

As shown in Table 2, the butyltins and inorganic tin were toxic to the two bacteria at about the same concentrations, but the orders of toxicity are slightly different between the two organisms. Application of one-way ANOVA support the relative orders of toxicity: for *P. fluorescens* SHC-6, the toxic order is TBT > DBT > Sn(IV) > MBT, and for *Serratia* sp. Gil-1 it is DBT ≥ TBT > Sn(IV) ≥ MBT. These results indicate that the toxicity of butyltins towards bacteria do not always decrease with degradation, as demonstrated for some other microorganisms [6,7,14,16,28].

EC_{50} values for tributylamine and tributylphosphate are significantly higher than those for TBT (Table 2). This suggests that the Sn moiety is important for TBT's toxicity.

Effects of butyltins and related chemicals on chemotaxis

In each experiment the average number of bacteria in control capillaries was set to zero, and the average number of bacteria in capillaries containing test chemicals was adjusted according to the equation: Relative number of cells in capillary = Actual number in capillary – Actual number in control capillary. A positive number indicates positive chemotaxis and a negative number indicates negative chemotaxis. The actual numbers in control capillaries are given with each figure.

TBT, DBT, MBT and $SnCl_4$ were negative chemotactic agents for *P. fluorescens* SHC-6. The concentrations of these compounds which were chemotactic (Fig. 1) were over four orders of magnitude less than the EC_{50} values (Table 2). At the lowest two concentrations of $SnCl_4$ and the lowest concentration of DBT used, the differences were not significant at the 95% confidence level as judged by one-way ANOVA. Throughout the paper, results of typical experiments are given, but each experiment was repeated two to four times.

At 8.4×10^{-7} and 8.4×10^{-6} M, TBT had no apparent effect on chemotaxis (Fig. 2), perhaps because it was toxic at these concentrations and cells were no longer capable of motility. There are at least two additional explanations. One is that prolonged clockwise rotation of the motor forces flagellar fila-

TABLE 2

EC_{50} of butyltins and related chemicals on viability of *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1 in chemotaxis medium after a 60-min exposure at 25°C

	EC_{50} (M) of*:					
	TBT	DBT	MBT	$SnCl_4$	tributylamine	tributylphosphate
P. fluorescens SHC-6	$^{a}1.8 \times 10^{-4} \pm 1.7 \times 10^{-4}$	$^{a}4.3 \times 10^{-4} \pm 1.5 \times 10^{-4}$	$^{b}2.4 \times 10^{-3} \pm 4.2 \times 10^{-4}$	$^{c}9.4 \times 10^{-4} \pm 3.0 \times 10^{-4}$	$^{b}1.1 \times 10^{-3} \pm 3.5 \times 10^{-4}$	$^{b}1.4 \times 10^{-3} \pm 6.6 \times 10^{-4}$
Serratia sp. Gil-1	$^{a}2.9 \times 10^{-4} \pm 1.7 \times 10^{-4}$	$^{a}1.6 \times 10^{-4} \pm 9.5 \times 10^{-5}$	$^{b}1.2 \times 10^{-3} \pm 6.4 \times 10^{-4}$	$^{b}1.1 \times 10^{-3} \pm 3.7 \times 10^{-4}$	$^{b}2.2 \times 10^{-3} \pm 4.4 \times 10^{-4}$	$^{c}5.6 \times 10^{-3} \pm 2.5 \times 10^{-4}$

* Each EC_{50} value was determined from three independent experiments.

[a–c] Means with different superscripts are significantly different at the 90% confidence interval.

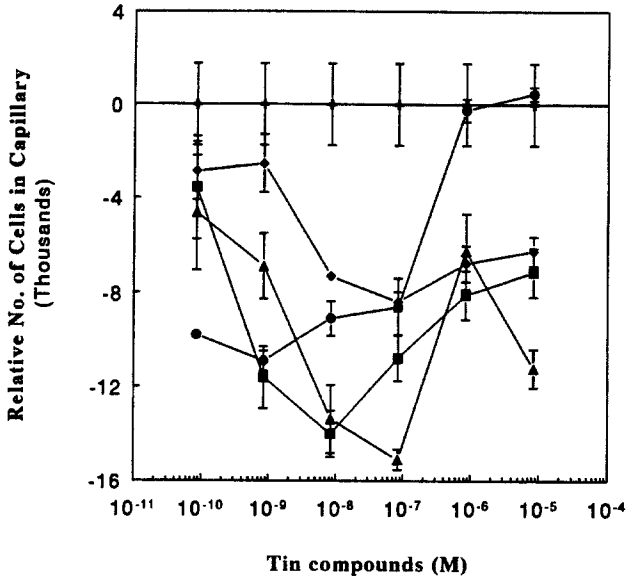

Fig. 1. Chemotactic effects of TBT, DBT, MBT and $SnCl_4$ on *P. fluorescens* SHC-6 at 25°C after 60 min incubation. The average number of cells in control capillaries was $2.2 \times 10^4 \pm 1.6 \times 10^3$. —+— control (no addition); —●— TBT; —■— DBT; —▲— MBT; —◆— $SnCl_4$. In this figure and in later figures, where no error bar is shown the bar falls within the symbol.

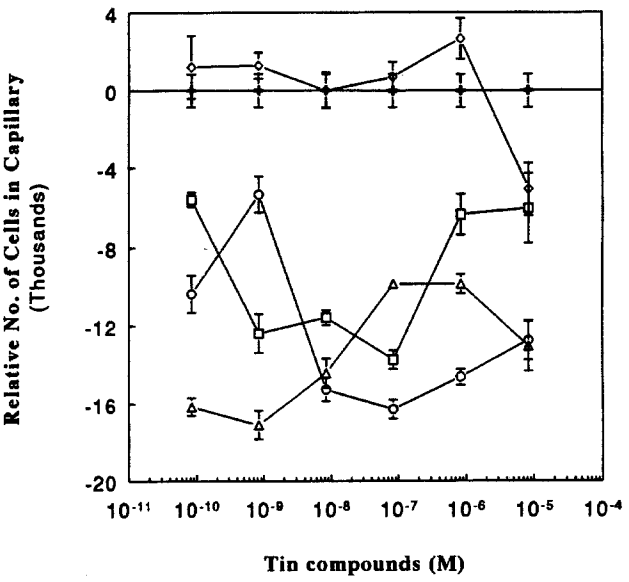

Fig. 2. Chemotactic effects of TBT, DBT, MBT and $SnCl_4$ on *Serratia* sp. Gil-1 at 25°C after 60 min incubation. The average number of cells in control capillaries was $2.2 \times 10^4 \pm 8.5 \times 10^2$. —+— control (no addition); —○— TBT; —□— DBT; —△— MBT; —◇— $SnCl_4$.

ments into right-handed helices that form a propulsive bundle. Commonly, repellents that increase clockwise rotation inhibit brief periods of counterclockwise rotation that disrupt the right-handed bundle; therefore, runs in the direction of higher repellent concentration are extended [33]. It is also possible that higher concentrations of TBT cause loss of flagella in *P. fluorescens* SHC-6. Li et al. [29] showed that wild-type *E. coli* lost motility in the presence of high concentrations of salts, carbohydrates, or low-molecular-weight alcohols. It is possible that when cells of *P. fluorescens* SHC-6 swam into the capillary containing higher (non-lethal, Table 2) concentrations of TBT some cells lost their flagella and were unable to swim out of the capillary, and so were counted in the viable count. Additional work is needed to explore these possibilities. One approach might use Phage χ to study motility in different sublethal concentrations of TBT because Phage χ requires functional rotating flagella for infection [24,40].

Figure 2 shows the chemotactic effect of butyltins and $SnCl_4$ on *Serratia* sp. Gil-1. The three butyltins were negative chemotactic agents at all concentrations tested, but $SnCl_4$ was not a chemotactic agent except at the highest concentration tested, 8.4×10^{-5}. As for *P. fluorescens* SHC-6, the butyltins were chemotactic at concentrations more than four orders of magnitude less than the EC_{50} concentrations.

The results with these two bacteria suggest that butyltins may repel motile aquatic bacteria. It is possible that one of TBT's functions in antifouling paints is to retard formation of a microbial biofilm. Such biofilms can stimulate development of barnacle and other fouling communities [12]. The results support the view that although a bacterial strain is resistant to a repellent it is still chemotactically repelled, and the mechanism for detection of repellents may be unrelated to deleterious effects of repellents [43].

Aspartate as a chemotactic attractant

Aspartate is a chemotactic attractant for several bacteria [11,34,37] and both *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1 were attracted to it (Fig. 3). The concentration of L-aspartate which elicited positive chemotaxis ranged from 10^{-1} to 10^{-6} M

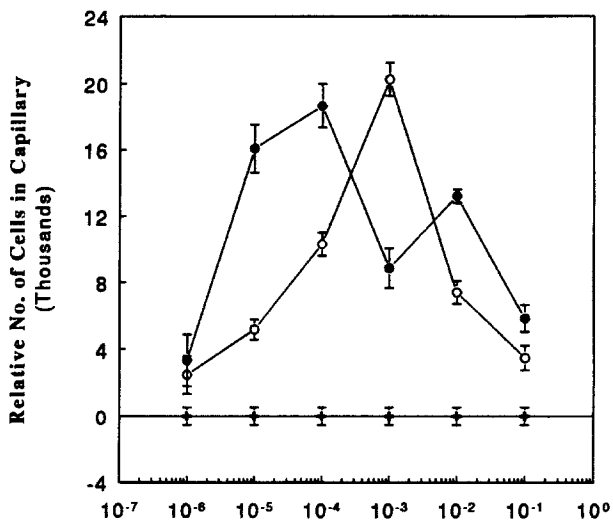

Fig. 3. Chemotactic effects of L-aspartate on *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1 at 25°C after 60 min incubation. The average number of cells in control capillaries was $1.8 \times 10^4 \pm 1.1 \times 10^3$ for SHC-6 and $7.9 \times 10^3 \pm 6.1 \times 10^2$ for Gil-1. —+— control (no aspartate); —●— *P. fluorescens* SHC-6 plus aspartate; —○— *Serratia* sp. Gil-1 plus aspartate.

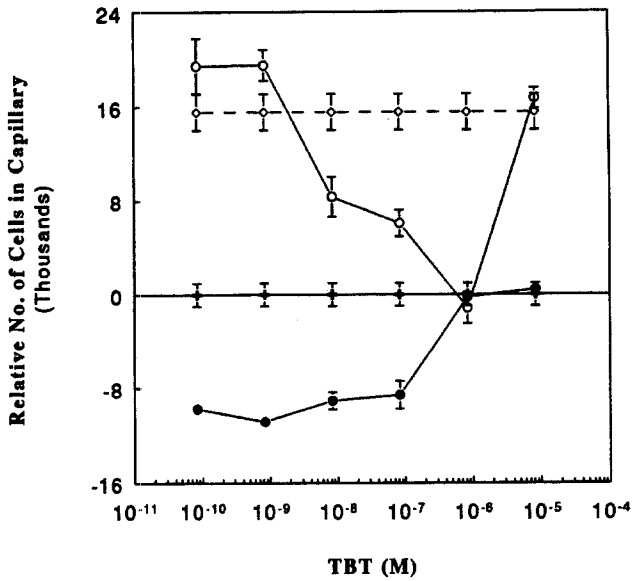

Fig. 4. Chemotactic effects of TBT and TBT plus 0.001 M L-aspartate on *P. fluorescens* SHC-6 at 25°C after 60 min incubation. The average number of cells in control capillaries was $2.1 \times 10^4 \pm 1.3 \times 10^3$. —+— control (no addition); --◇-- aspartate; —●— TBT; —○— TBT and aspartate.

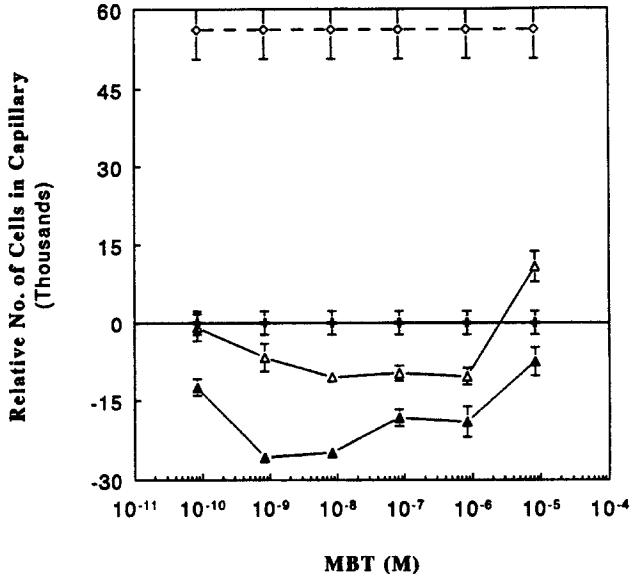

Fig. 6. Chemotactic effects of MBT and MBT plus 0.001 M L-aspartate on *P. fluorescens* SHC-6 at 25°C after 60 min incubation. The average number of cells in control capillaries was $4.2 \times 10^4 \pm 2.3 \times 10^3$. —+— control (no addition); --◇-- aspartate; —▲— MBT; —△— MBT and aspartate.

and the differences were significant at the 95% confidence interval. At 10^{-1} M, L-aspartate was a weak attractant. A number of compounds which are attractants to bacteria at lower concentrations are repellents at higher concentrations [43].

All three butyltins were examined for their effect on chemotaxis to aspartate, using aspartate at 10^{-3} M. All butyltins: TBT, DBT and MBT negated the positive chemotactic effect of aspartate on *P. fluorescens* SHC-6 (Figs 4–6), but they did not negate the positive chemotactic effect of aspartate on *Serratia* sp. Gil-1. Results are shown only for TBT (Fig. 7). The different responses of the two organisms to butyltins in the presence of aspartate suggests that they have different chemotaxis response systems for aspartate or for butyltins.

The toxic effect of butyltins is related to their hydrophobicity which may involve interaction between the butyltins and the surface of the cell membrane [8,17]. Based on K_{ow}

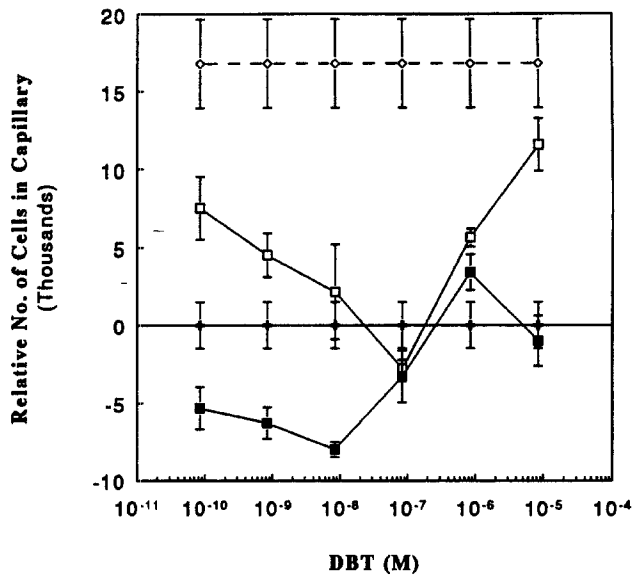

Fig. 5. Chemotactic effects of DBT and DBT plus 0.001 M L-aspartate on *P. fluorescens* SHC-6 at 25°C after 60 min incubation. The average number of cells in control capillaries was $2.2 \times 10^4 \pm 1.7 \times 10^3$. —+— control (no addition); --◇-- aspartate; —■— DBT; —□— DBT and aspartate.

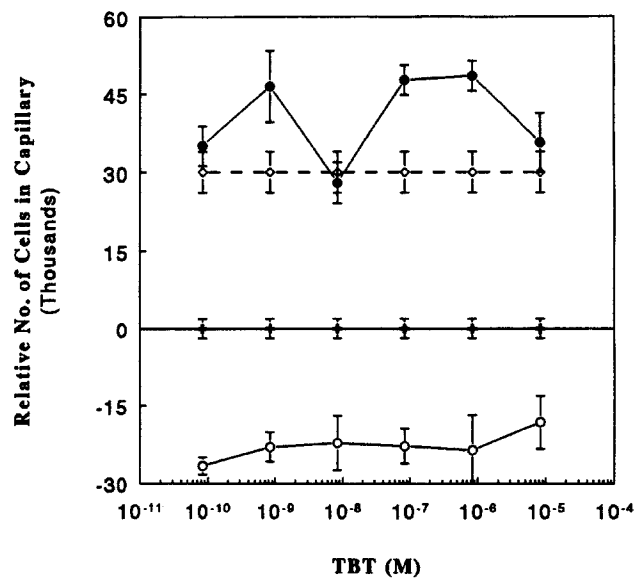

Fig. 7. Chemotactic effects of TBT and TBT plus 0.001 M L-aspartate on *Serratia* Gil-1 at 25°C after 60 min incubation. The average number of cells in control capillaries was $2.7 \times 10^4 \pm 1.9 \times 10^3$. —+— control (no addition); --◇-- aspartate; —○— TBT; —●— TBT and aspartate.

value (octanol/water partition ratios), TBT is considered moderately lipophilic [27,31] and it may penetrate the cell membrane. TBT can adhere to and denature membrane proteins [45]. It is not known whether there are different receptors for L-aspartate and butyltins, or if both bind to a single receptor. If butyltins and L-aspartate are detected by different receptors, the signals generated and transduced may be conflicting, so butyltins would decrease the positive chemotactic response to aspartate, or aspartate would overcome the negative chemotactic response to butyltins. If butyltins penetrate the cell membrane or react with its surface, they may influence the action of transducing proteins, the flagellar switch proteins or the flagellar motor, either by associating with membrane phospholipids in the immediate environment of transducing proteins or by binding to the proteins themselves [45]. The mechanisms and interactions are complex and additional studies are needed to understand them.

Related compounds

Tributylamine and tributylphosphate are toxic to *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1, although they are less toxic than TBT (Table 2). Neither chemical had a chemotactic effect on either organism at concentrations from 10^{-4} to 10^{-10} M (data not shown). This indicates that the Sn moiety contributes significantly to the toxicity and the negative chemotactic effects of butyltins.

Similarly, $SnCl_4$ was less toxic to these organisms than TBT or DBT (Table 2). It was not chemotactic for *Serratia* sp. Gil-1 at a concentration less than 8.4×10^{-6} M (Fig. 2) and was not effective at 8.4×10^{-10} M or less as a chemotactic agent for *P. fluorescens* SHC-6 while butyltins were effective at those concentrations (Fig. 1). Therefore, the butyl-moieties of butyltins contribute to their toxicity and chemotactic properties.

The mechanisms of bacterial chemotaxis are complex and are not completely understood. But chemotactic behavior certainly provides a selective advantage for motile bacteria. Motile bacteria possess a mechanism for detecting and removing themselves from lethal as well as sublethal concentrations of butyltins. This study shows that butyltins are strong chemotactic repellents to the two marine motile bacteria, *P. fluorescens* SHC-6 and *Serratia* sp. Gil-1, at concentrations which are over four orders of magnitude less than their EC_{50} values. Butyltins may have common properties of negative chemotaxis. TBT is a strong antifouling agent. Its negative chemotactic effect may contribute to its antifouling activity by inhibiting the development of a primary fouling layer containing bacteria. If butyltins have the same negative chemotactic property towards other motile aquatic bacteria at sublethal concentrations, if negating microbial chemotaxis contributes to antifouling, and if the adverse effects of butyltins can be decreased greatly at low concentrations, it may be possible to formulate antifouling paints which are less toxic to non-target macroorganisms and to re-evaluate the regulation and use of butyltins as antifouling agents.

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