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# Toxicity of Butyltin, Phenyltin and Inorganic Tin Compounds to Sulfate-reducing Bacteria Isolated from Anoxic Marine Sediments

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**The toxicity of butyltin, phenyltin and inorganic tin compounds to three pure strains of sulfate-reducing bacteria (SRB), isolated from a tributyltin (TBT)-polluted sediment, was determined. The isolated strains were identified as belonging to the genus *Desulfovibrio*. A new toxicological index ($GR_{25}$) was developed to assay the toxicity of organotin compounds. Deleterious effects on suspended anaerobic cell cultures were observed for concentrations ranging between 500 and 600 $\mu$M for tin tetrachloride, 55 and 260 $\mu$M for triorganotins, 30 and 90 $\mu$M for diorganotins, and 1 and 6 $\mu$M for mono-organotins. Whereas the number of substituents influenced the toxicity of organotins, the type of substituent (butyl or phenyl) proved to have little or no impact. Trisubstituted compounds (tributyl- and triphenyl-tin) were less toxic to these strains of SRB than the monosubstituted forms (monobutyl- and monophenyl-tin). This is the opposite trend to that currently reported for aerobic organisms. Under the given anoxic conditions, the toxicity of organotin compounds obtained yielded a significant negative correlation with the total surface area (TSA) of the tested molecules. Comparison of the TBT toxicity data observed for different microbial groups suggests that the tolerance of bacteria to organotin compounds might be related to organotin–cell wall interactions as well as to aerobic or anaerobic metabolise pathways. Copyright © 2000 John Wiley & Sons, Ltd.**

**Keywords: organotin compounds; sulfate-reducing bacteria; *Desulfovibrio*; structure–toxicity relationships; total surface area (TSA); tolerance**

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## 1 INTRODUCTION

Organotin compounds (OTs) are used by industry as plastics stabilizers and biocidal agents, etc.[1] The biocidal applications generate direct entries into the environment and can result in acute contamination of aquatic environments around the world.[2,3] Tributyltin (TBT) is one of the most efficient and most toxic components added to antifouling paints. Its dispersion in the environment has caused serious deleterious effects on shellfish, even at very low concentrations (ng $l^{-1}$).[4–8] The environmental hazards associated with TBT have provoked regulation and restricted use of TBT-containing antifouling paints. Despite of the fact that direct anthropogenic input should have been (in principle) reduced, the decrease in contamination of ecosystems has not been clearly demonstrated in every area. Indeed, due to their weak degradability in anoxic sediments, butyltin compounds remain a potential source of contamination for aquatic environments.[9–11] Triphenyltin (TPheT) is used in agriculture as acaricide and fungicide and, to a lesser extent, in antifouling paints. However, its toxic effects are not fully known.[12,13]

It is generally believed that the toxicity of organotin compounds increases with increasing size of the alkyl group of the OT molecule and with increasing substitution of the tin atom.[14,15] Previous studies on a wide variety of organisms (eukaryotes and prokaryotes) showed that trisubstituted tin compounds were more toxic than their

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tetra- and mono-substituted counterparts.[12,13,16–20] Some of this work also demonstrated that OT toxicity was dependent on the nature of the substitutents.[12,18] Various parameters have been tested to estimate relationships between molecular structure and biological activity, especially those that represent the hydrophobicity of the molecules. For example, the Hansh constants or the octanol/water partition coefficients ($K_{ow}$) have been shown to be correlated with the toxicity of OTs.[13,21–23] A good correlation between the toxicity of organotin compounds and a topological parameter such as the total surface area (TSA) of the molecules has been demonstrated as well.[17]

Most results published to date on toxicity studies were obtained with aerobic bacteria. Few studies have focused on anaerobic bacteria, even though the highest concentrations of tin compounds are usually found in the anoxic part of sediments. Belay *et al.*[24] showed that methyltins were more toxic to anaerobes than butyltins and that TBT was the least toxic compound for methanogenic and sulfate-reducing bacteria. Likewise, Boopathy and Daniels[25] demonstrated that, in contrast to results obtained with aerobes, the toxicity of OTs to methanogenic bacteria decreased as the TSA of the molecules increased.

Generally, in marine and brackish anoxic environments, sulfate-reducing bacteria (SRB) represent a major part of the total bacterial population and contribute largely to organic matter degradation processes.[26] In the TBT-contaminated coastal sediments, SRBs are often in contact with high levels of tin compounds and these bacteria are believed to be mainly responsible for their biotransformation.[27–29] However, their tolerance of OTs has been poorly studied and should be further investigated since this group of bacteria may affect the persistence or the transformation of tin chemical species in the aquatic environment.

The present work evaluates the toxicity of tin compounds to *Desulfovibrio* strains isolated from a TBT-polluted sediment. A new toxicological index has been developed for SRB cultures grown in liquid media, in order to minimize possible interferences between metals and gelling agents and also to evaluate toxic effects at low OT concentrations. The toxicity values obtained for butyl- and phenyltin compounds were compared with the TSA to determine a structure–toxicity relationship. The present study and other contributions[13,17,25] clearly demonstrate opposite correlations between aerobic and anaerobic metabolic pathways with respect to organotin compounds. A comparison of TBT toxicity for different bacterial groups demonstrates the role of cell-wall structure (Gram-positive or Gram-negative staining) in bacterial tolerance of organotin compounds.

## 2 MATERIAL AND METHODS

### 2.1 Sample collection

Bacterial strains were isolated from sediments sampled in Arcachon Bay, France. Arcachon Bay is a lagoon located in the south of the Atlantic French coast. The water salinity ranges between 20 and 35 $g^{-1}$. The deleterious effects of TBT pollution were demonstrated in this ecosystem in the early 1980s.

Sediment samples were collected in Arcachon harbor, where bacteria have long been in contact with high levels of TO compounds. Samples were collected with a Plexiglas hand-corer and kept in the dark at 4 ° C before isolation of the bacteria. Muddy anoxic sediments contained a sulfide concentration (free $H_2S$ + acid volatile sulfides) of about 50 mM and average TBT concentrations of 150 ng $g^{-1}$ (as Sn determined by GC/FPD).[11,30]

### 2.2 Isolation and identification of strains of sulfate-reducing bacteria (SRB)

SRB enrichments were obtained at a depth of 5–10 cm in the sediment and were grown in a synthetic medium according to Pfennig *et al.*[31] after three consecutive transfers.

Sulfate-reducing bacteria were isolated after enrichment in the same synthetic media. Strains were isolated by successive dilution agar shaking series in anaerobic conditions using the Hungate technique. Pure strains were maintained in liquid culture in 120 ml bottles sealed with rubber stoppers. The purity of the strains was checked by phase-contrast microscopy (Olympus model BH-2) and by growth tests (both aerobically and anaerobically) in sulfate-free TYG medium. The strains (PA 2803, PA 2804, PA 2805) were identified by morphological observations and biochemical growth tests. The morphology of the bacteria was examined by phase-contrast microscopy. Metabolic tests were performed[32] in order to determine the utilization of various carbon and energy sources, the utilization of diverse electron acceptors and the potential fermentative growth of bacteria.

 

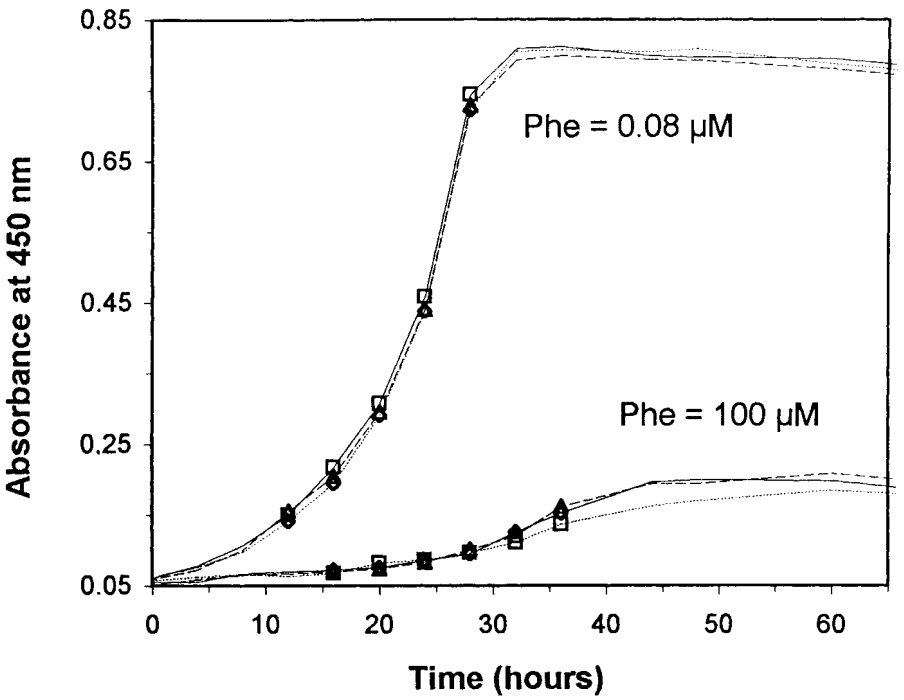


**Figure 1** Effect of two concentrations of monophenyltin (Phe) on the growth of *Desulfovibrio* strain PA 2803. Each curve represents a replicate culture. Absorbances were measured at 450 nm every 4 h. Symbols represent the values measured during the exponential phase of growth which were used to calculate the growth rate for each replicate.

## 2.3 Tin compounds

Tin compounds [$SnCl_4$, ($C_4$ $H_9$) $SnCl_3$, ($C_4$ $H_9$)$_2$ $SnCl_2$, ($C_4$ $H_9$)$_3$ SnCl, ($C_6$ $H_5$) $SnCl_3$, ($C_6$ $H_5$)$_2$ $SnCl_2$, ($C_6$ $H_9$)$_3$ SnCl] were purchased from Strem Chemicals, France, and were used without further purification. Stock solutions were prepared in ethanol (100%). Final ethanol concentrations in test-tubes were less than 10 mM. Reference cultures without tin compounds were grown in the presence of 10 mM ethanol to determine the possible stimulation or inhibition effects.

## 2.4 Selection of a toxicological index

In toxicity studies, various methods can be used to determine the level of a pollutant that might affect micro-organisms adversely. Each experimental approach aims to reflect real processes taking place in the environment. However, experimental conditions are generally far from field conditions and may lead to severe limitations in the results obtained. We have therefore developed a rapid and simple method to evaluate OT toxicity under anoxic conditions.

Previous work was usually based on the use of a solidified culture medium to test the toxicity of organotin compounds to bacteria[33,34] or yeasts.[16] However, it is well known that interactions (e.g. chelation, complexation) between compounds of tin (or other metals) and gelling agents can occur and affect the general results obtained.[19,35,36] Homogeneity of the gel media with respect to tin concentration may also be a problem. In order to bypass some of these inconveniences, we have chosen to develop our test in a liquid culture medium.

Different conditions and indexes have been defined to express the toxicity of a molecule. Inhibiting concentrations of the compound are often used in such experiments. One common inhibition index is the minimum inhibiting concentration (MIC), which is the lowest concentration of OT that causes a complete inhibition of growth. This approach has been used for trialkyltin chlorides[37] or TBT[34] and the index values (MIC values) obtained range between 1 and 3000 $\mu g^{-1}$

 

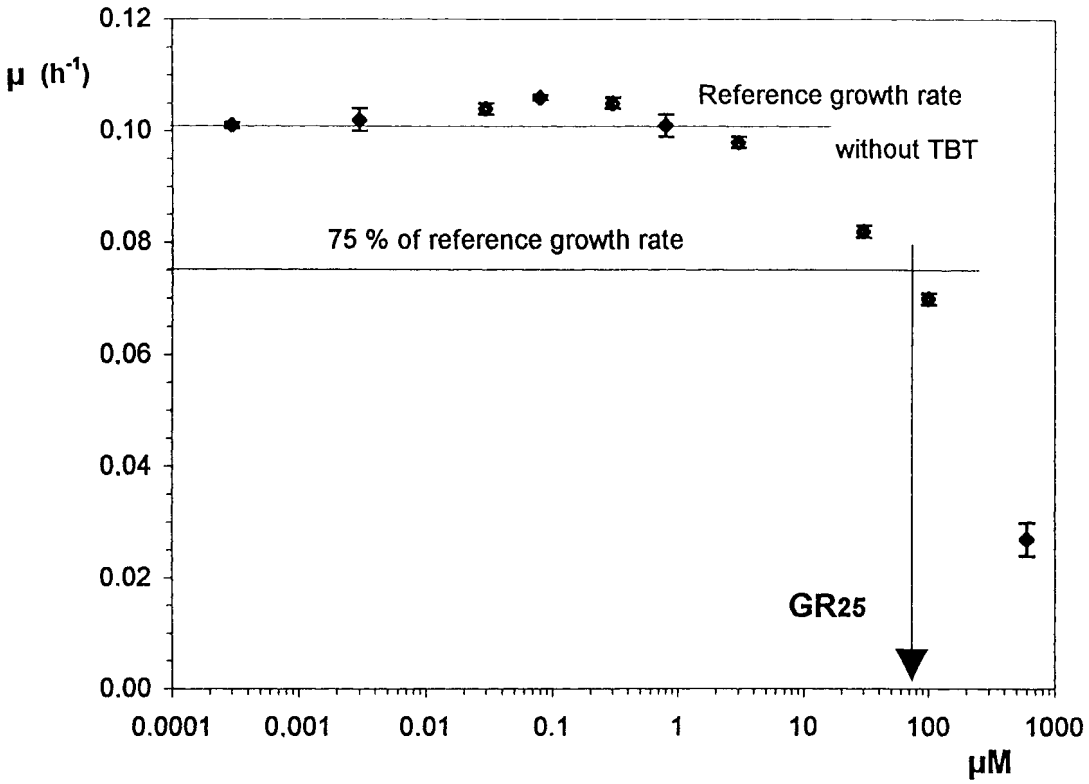


**Figure 2** Example of toxicological index $GR_{25}$ determination from mean growth rate ($\mu$) for PA 2803 subjected to different tributyltin concentrations. Vertical bars indicate the confidence interval (95%).

for Clostridia, Pseudomonads and Enterobacteria. Such elevated concentrations, however, do not correspond to those usually found in the environment. Furthermore, spiking of such high levels of OTs in cultures may lead to precipitation and complexation of the tin compounds. This phenomenon is particularly likely with high-ionic-strength media.[38] Toxic effects below the lethal concentrations can be recorded by determining changes in bacterial metabolic activity or the total growth in liquid cultures. Jonas *et al.*[39] compared different methods to determine the toxic effects of metal and organometal to microbial communities. Recording the incorporation of [$^3$H]thymidine appeared to be more sensitive than viability tests. This test was also more applicable to a large diversity of bacterial communities compared to results obtained with [$^{14}$C]glutamate incorporation (as a substrate). Belay *et al.*[24] studied the effect of alkyltin compounds on methane ($CH_4$) production by methanogenic bacteria. These methods yield good results but an easier method is the direct measurement of the culture growth. This measurement most typically relies on the determination of the culture turbidity by direct recording of the absorbance.[24,25,37] It is generally accepted that the final state of the culture is obtained just after reaching the stationary phase of population growth.

Boopathy and Daniels[25] have used an $IC_{50}$ index corresponding to the concentration of organotin compounds resulting in a decrease of half of the final absorbance compared to that of control cultures grown in the absence of the OT. In such experiments, the maximal absorbance is more and more slowly reached as the concentration of the pollutant increases. This time dependence makes for a difficult comparison of the data obtained.

To avoid that, we have recorded and calculated the growth rates in liquid media for the different tin concentrations studied. Further, the growth rates can be determined earlier than the maximal absorbance values.

Under the experimental conditions to be studied, the bacterial cultures were distributed in three

 

**Table 1** Electron donors and acceptors utilized by SRB strains PA 2803, PA 2804 and PA 2805 isolated from Arcachon harbor sediments

| | Utilization by | | |
|---|---|---|---|
| Electron donor or acceptor | PA 2803 | PA 2804 | PA 2805 |
| Electron donor[a] | | | |
| $H_2$ ($10^5$ Pa) + acetate (10 mM) | +[c] | + | −[c] |
| Lactate (10 mM) | + | + | + |
| Acetate (10 mM) | − | − | − |
| Propionate (10 mM) | − | − | − |
| Butyrate (10 mM) | − | − | − |
| Pyruvate (10 mM) | + | + | + |
| Malate (10 mM) | + | + | + |
| Fumarate (10 mM) | + | + | − |
| Succinate (10 mM) | + | + | − |
| Benzoate (5 mM) | − | − | − |
| Ethanol (10 mM) | + | + | + |
| Electron acceptor[b] | | | |
| Thiosulfate (10 mM) | + | + | + |
| Sulfur | + | + | − |
| Nitrate (10 mM) | + | (+)[c] | − |

[a] Electron donors were tested in the presence of 20 mM sulfate.
[b] Electron acceptors were tested in presence of 10 mM lactate as a source of carbon and energy.
[c] +, Good growth; (+), slight growth; −, no growth.

Hungate tubes for each tin compound and the concentration was tested. The growth of the culture in the previously cited synthetic medium was checked every four hours by direct measurement of the absorbance of the culture at 450 nm with a spectrophotometer (Spectronic 20D; Milton Roy) (Fig. 1). The exponential growth rate was estimated from the slope of the regression obtained by plotting absorbance (on a log scale) versus time. The mean growth rate was determined from nine *Desulfovibrio* cultures (three strains with three different replications). The growth rates were plotted as a function of the tin compound concentration for all the different strains and molecules tested (an example is presented in Fig. 2). The toxicological index ($GR_{25}$) was defined graphically as the concentration of the tin compound which resulted in a growth rate 25% lower than the reference value obtained in the absence of tin for the same cultures under identical conditions (Fig. 2). The 25% value was chosen because OT concentrations leading to a 50% decrease of the growth rate were too high, and weak fluctuations of the OT concentration yielded rapid variations in growth rates. Moreover, with such elevated OT concentrations the absence of interactions between these compounds and the culture media could not be confirmed.

# 3 RESULTS

## 3.1 Identification of isolates

Morphologically, the cells of the three strains PA 2803, PA 2804 and PA 2805 appeared to be motile, curved rods. PA 2803 and PA 2804 were 0.4 $\mu$m wide and 1.5 –3 $\mu$m long. PA 2805 was slightly larger, 0.5 $\mu$m wide and 1.5 –4 $\mu$m long. For the three strains, sporulation was never observed and Gram staining was negative. The substrates tested as the possible energy and carbon sources are listed in Table 1. The three strains grew well on lactate, pyruvate, malate and ethanol but not on acetate. According to these characteristics, the three strains were considered as members of the genus *Desulfovibrio*.

## 3.2 Method

The mean growth rates obtained for reference cultures grown without tin compounds were $0.102\ h^{-1}$ in the presence of 10 mM ethanol and $0.100\ h^{-1}$ without ethanol. Therefore, this ethanol concentration did not significantly stimulate or inhibit microbial growth of these SRB strains.

The determined values of the toxicological index ($GR_{25}$) were highly reproducible. The confidence

 

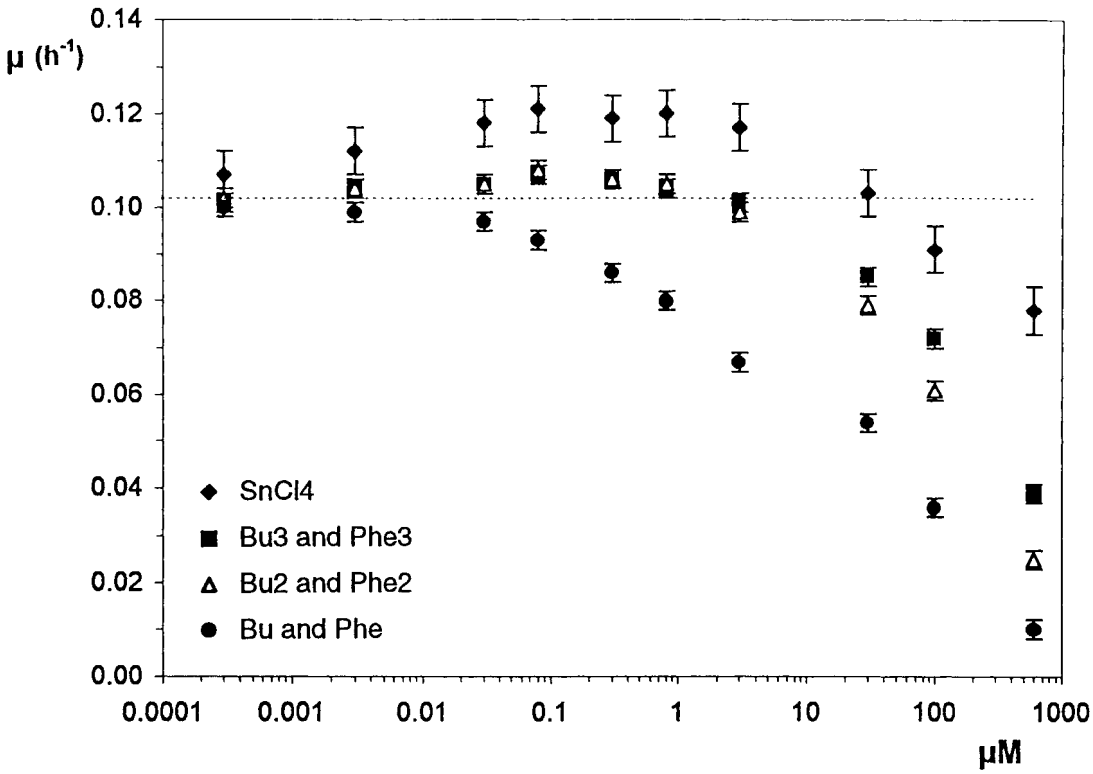


**Figure 3** Mean growth rates ($\mu$) for *Desulfovibrio* strains PA 2803, PA 2804 and PA 2805 subjected to different concentrations of inorganic Sn, mono-, di- or tri-butyltin, or phenyltin. The broken line represents the reference growth rate of cultures not contaminated by Sn and grown in the same conditions. Vertical bars indicate the confidence intervals (95%). Bu, butyltin; $Bu_2$, dibutyltin; $Bu_3$, tributyltin; Phe, phenyltin; $Phe_2$, diphenyltin; $Phe_3$, triphenyltin.

intervals (95%) calculated with the three replicates were always less than $\pm 0.008\ h^{-1}$ (data not shown).

## 3.3 Toxicity of tin compounds

Figure 3 presents mean growth rates for three *Desulfovibrio* strains as a function of the concentration of several tin compounds. Values were very similar for tributyltin and triphenyltin, dibutyltin and diphenyltin, and monobutyltin and monophenyltin respectively. Therefore, the mean growth rates obtained for mono-, di- and tri-substituted OTs were grouped under the same symbols in the Figure. A deleterious effect on the SRB cultures was observed at 100 $\mu$M for tin tetrachloride, 10 $\mu$M for tri- and di-substituted OTs and 0.1 $\mu$M for monosubstituted OTs. Inorganic tin, $SnCl_4$, was the least toxic compound for the strains tested. Growth rates in the presence of tri- or di-OTs were similar with concentrations less than 30 $\mu$M. At concentrations above 30 $\mu$M, the disubstituted compounds appeared to be more toxic. Monobutyltin and monophenyltin exhibited the highest toxicity among the OTs tested.

For all the tin compounds tested, with the exception of the monosubstituted series, a slight increase in the growth rate was observed for low levels of OT inoculation. When the cultures were spiked with $SnCl_4$ at concentrations ranging between 3 nM and 3 $\mu$M, the growth rates were higher ($0.118–0.120\ h^{-1}$) than in reference cultures grown without tin ($0.102\ h^{-1}$). Similarly, cultures inoculated with 0.08 $\mu$M di- or tri-substituted OTs showed better growth rates ($0.107\ h^{-1}$) than the cultures containing no tin compound ($0.102\ h^{-1}$).

The $GR_{25}$ values determined for the three *Desulfovibrio* strains and for each organotin compound are presented in Table 2. Values ranged between 500 and 600 $\mu$M for $SnCl_4$, 55 and 260 $\mu$M for triorganotins, 30 and 90 $\mu$M for diorganotins, and 1 and 6 $\mu$M for mono-organotins.

Statistical analysis of the data did not show any

 

**Table 2** $GR_{25}$values ($\mu$M) determined for three *Desulfovibrio* strains from their growth rates (mean of triplicate cultures) plotted as a function of Tin compound concentration

| | | Desulfovibrio | | |
|---|---|---|---|---|
| Compound | Formula | PA 2803 | PA 2804 | PA 2805 |
| Tributyltin | $(C_4\ H_9)_3\ SnCl$ | 70 | 55 | 170 |
| Triphenyltin | $(C_5\ H_6)_3\ SnCl$ | 70 | 60 | 260 |
| Dibutyltin | $(C_4\ H_9)_2\ SnCl_2$ | 30 | 40 | 70 |
| Diphenyltin | $(C_5\ H_6)_2\ SnCl_2$ | 40 | 50 | 90 |
| Monobutyltin | $(C_4\ H_9)\ SnCl_3$ | 1.4 | 4 | 1.6 |
| Monophenyltin | $(C_5\ H_6)\ SnCl_3$ | 0.3 | 6 | 1.2 |
| Inorganic tin | $SnCl_4$ | 500 | 600 | 500 |

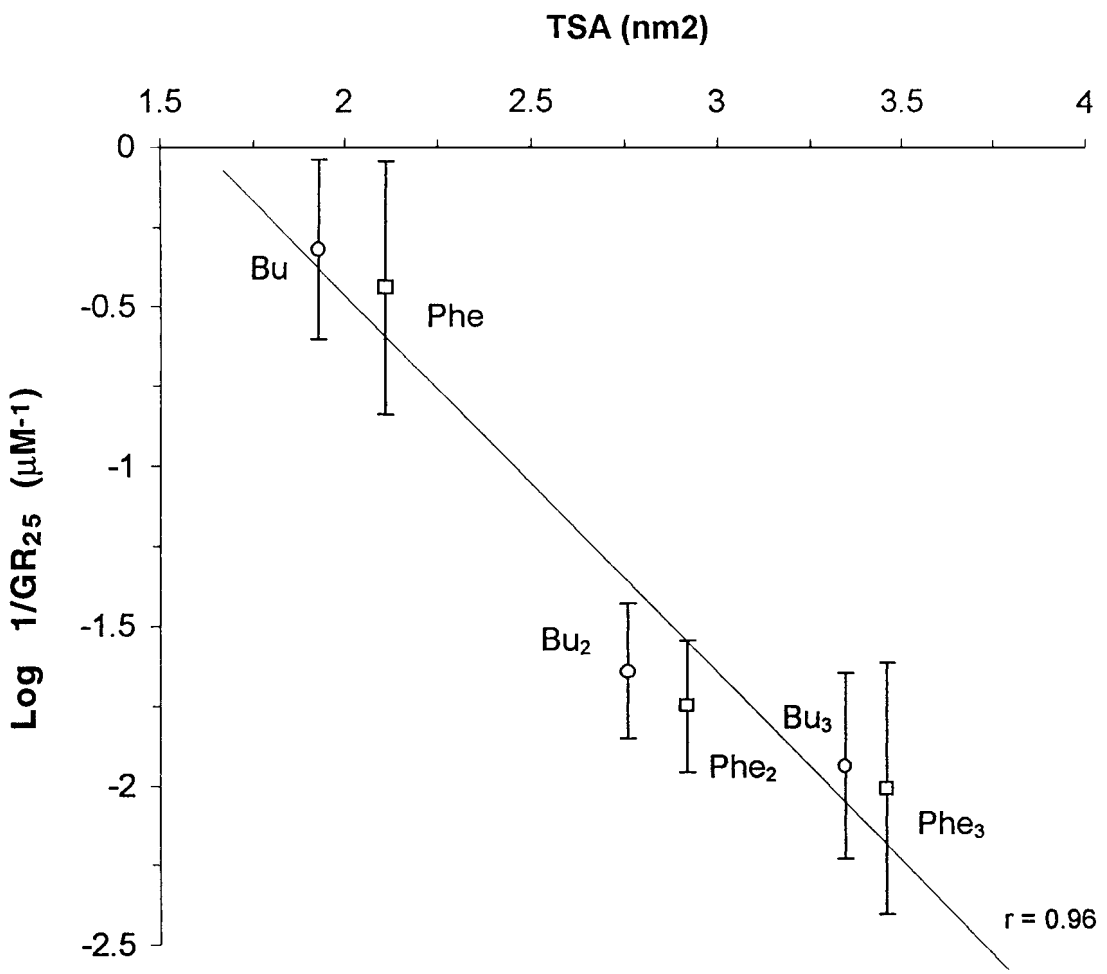


**Figure 4** Relationship between mean organotin toxicity [$\log(1/GR_{25})$] and compound TSA for *Desulfovibrio* PA 2803, PA 2804 and PA 2805 strains. Vertical bars indicate the confidence intervals (95%). Bu, butyltin; $Bu_2$, dibutyltin; $Bu_3$, tributyltin; Phe, phenyltin; $Phe_2$, diphenyltin; $Phe_3$, triphenyltin.

 

**Table 3** TBT minimum inhibitory concentrations (MICs) for different bacterial groups

| Bacterium | MIC | Metabolism | Cell wall | Refs |
|---|---|---|---|---|
| *Clostridium* | 1.5–3.7 $\mu$M | Anaerobe | Gram + | 25 |
| *Bacillus* | 10 $\mu$M | Aerobe | Gram + | 43[a] |
| $CH_4$-producing | 1 mM | Anaerobe | Archeaebacteria | 4 |
| *Desulfovibrio* (SRB) | >2 mM | Anaerobe | Gram − | 4[b] |
| *Pseudomonas* | 0.07–3 mM | Aerobe | Gram − | 25 |
| *Enterobacteriaceae* | 0.9–3 mM | Facultative aerobe | Gram − | 25, 43 |

[a] Unpublished data
[b] Extrapolation from our present results.

significant difference of toxicity within the different family of tri-, di- and mono-substituted compounds. However, a marked difference in toxicity between the mono-, di-, and tri-substituted OTs was demonstrated. The decreasing order of toxicity for the SRBs tested was then established to be as follows: monobutyl- and monophenyl-tin, dibutyl- and diphenyl-tin, tributyl- and triphenyltin, inorganic tin.

# 4 DISCUSSION

## 4.1 Toxicity of organotin compounds

Contrary to previously published results for aerobic bacteria,[12,16,17,19,20,23,39] in the present study tributyl- and triphenyl-tin did not appear to be the most toxic organotin compounds for the three strains of SRB. Under the experimental conditions employed here, monosubstituted compounds such as monobutyl- and monophenyl-tin compounds displayed the most pronounced inhibition of the bacterial growth. These results are in agreement with those of Belay *et al.*,[24] who have recently demonstrated that monosubstituted (methyl or butyl derivatives) were the most toxic tin compounds for methanogenic bacteria and SRB belonging to the *Desulfovibrio* genus.

If the number of substituents made a significant difference on the level of the toxicity observed (Fig. 3, Table 2), the type of substituent (i.e. phenyl vs butyl) had only a minor impact. This is in contrast with the observations of Yamada *et al.*,[37] who reported that the toxicity of trialkyltin chlorides towards yeasts, fungi and aerobic bacteria increased with the size of the substituted group in the following order: trimethyltin > triethyltin > tripropyltin > tributyltin.

### 4.2 Structure–toxicity relationships

Since the degree of toxicity caused by the tested tin compounds appeared to be related to the number of organic substituents, the possible occurrence of a structure–toxicity relationship was examined. In this work, the toxicity of organotin compounds to SRB, expressed as the logarithm of ($1/GR_{25}$), was plotted against the TSA (Fig. 4). The TSA values used in this graph were obtained from Laughlin *et al.*[13] and Boopathy and Daniels.[25] For the SRB strains tested, an inverse linear relationship ($r^2 = 0.96$) was obtained between the two parameters, i.e. the toxicity of the tested OTs increased as the TSA decreased. Boopathy and Daniels[25] reported a similar toxicity–TSA correlation for methanogenic bacteria.

However, the present results, obtained with strictly anaerobic bacteria, do not agree with those obtained with aerobic eukaryotes[13,17] or with the facultative aerobic *Escherichia coli*.[25] However, it can be noted that recent studies[40–42] did not find any significant correlation between OT toxicity and different molecular descriptors. The occurrence of such a relationship, which could be fortuitous, remains controversial, particularly regarding the different bacterial groups.

Without using a toxicity–structure relationship, Yamada *et al.*[37] reported that the toxicity of trialkyltin chlorides to yeasts, fungi and aerobic bacteria increased in the following order: trimethyltin, triethyltin, tripropyltin and tributyltin (i.e. increasing toxicity with increasing TSA). Previous results showed that the trialkyltins were more toxic to yeasts than the mono- and di-alkyltins.[16]

The greater toxicity of monosubstituted tin compounds than of the tri- or di- substituted forms

 

has only been demonstrated for the strictly anaerobic micro-organisms (sulfate-reducing or methanogenic bacteria) that have been tested to date.

### 4.3 Bacterial tolerance of organotin compounds

Previous studies and the present results seem to argue for the influence of respiratory metabolism in either more or less bacterial tolerance of OTs. Table 3 presents a review of the minimum inhibiting concentrations (MICs) of TBT for various groups of bacteria. The aerobic or anaerobic metabolism involved and the Gram staining are also presented. From this table, Gram-positive bacteria appear to be more sensitive to TBT than Gram-negative bacteria, regardless of their respective type of metabolism. Argese *et al.*[42] have reported that triorganotins may act at the membrane level and are harmful to the energy-coupled processes. We speculate that the toxicity of a trisubstituted tin compound to a bacterium would also depend on its interaction with the structural and functional differences of the cell walls and not only on the type of the metabolism (aerobic or anaerobic). Both may act at different levels, resulting in the final expression of OT toxicity. More data are required in order to verify this hypothesis.

### 4.4 Effect of inorganic tin on bacterial growth

As presented in Fig. 3, the growth of the isolated *Desulfovibrio* strains was significantly enhanced in the presence of 0.003–3 $\mu$M inorganic tin or 0.08 $\mu$M tri- or di-organotin. No increase in growth rate was observed in the control culture without tin (the broken line in Fig. 3). This is the first evidence of a positive effect of low concentrations of inorganic or organic tin compounds on the growth of a bacterium, perhaps as the result of an adaptation of these *Desulfovibrio* strains to tin-contaminated sediments.

## 5 CONCLUSION

Organotin compounds degrade slowly in sediments, in the anaerobic part of which most are accumulated. The results presented in this paper demonstrate the high toxicity of monosubstituted compounds to sulfate-reducing bacteria, suggesting that in anoxic sediments biodegradation of the trisubstituted OTs might be limited by the resulting production of more toxic monosubstituted compounds.

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