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Effects of organotin and organolead compounds on yeasts

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SUMMARY

Four methods were used to screen nine organotin and two organolead compounds for toxicity to 29 yeasts, representing 10 genera. Center well diffusion plates were useful in comparing the sensitivity of yeasts to the most toxic organometals but were not useful for comparisons between compounds because of differences in diffusion rates and lack of sensitivity. Two-layer diffusion plates (density gradient plates) were also of limited use for comparisons between compounds but provided quantitative information on toxicity and allowed comparisons between organisms. Two-dimensional diffusion plates were useful for estimating the effect of pH on organometal toxicity. Release of K^+ from cell suspensions measured using a K^+-electrode provided quantitative information and allowed comparisons between compounds and organisms. The presence of 3% NaCl in cell suspensions decreased the rates and extent of organotin-induced K^+ release. Yeasts varied in their sensitivity from strain to strain, but tributyltin was the most toxic compound tested. Mono- and dimethyltins were the least toxic. Triphenyltin, dibutyltin, monobutyltin, trimethyltin, triethyltin, diethyllead, diethyltin, and dimethylleads showed intermediate toxicity, but triphenyltin and monobutyltin were the most toxic among the group.

INTRODUCTION

Heavy metals are toxic to a variety of microorganisms, and organometals are generally more toxic than inorganic forms of the same metal. Moreover, some organometals are more volatile and more soluble than the elemental metal or cationic forms of the metal. Thus, organometals have altered mobility in the environment as well as altered toxicity [7,8].

In the environment organoleads are of concern because of the use of tetraethyllead as a gasoline additive and because such compounds can cause changes in natural microbial populations [21]. A number of organotins are used in chemical processes, in consumer goods and as biocidal agents [27].

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Methyltins are of interest because they can be produced by microbial action [4,6,9,14,15,18], and tributyltin is of concern because it is a toxic component of antifouling paints [5]. Methyltins and tributyltin are toxic to pure microbial cultures [16,17,19,31], indicator organisms [23] and also natural populations [24]. Nevertheless, the action of organoleads and organotins on microbial cells is poorly understood, and information on their toxicity to yeasts is deficient.

The present work was undertaken in order to compare several methods for their application in screening organometals for toxicity and in order to assess the susceptibility of yeasts to selected organoleads and organotins.

MATERIALS AND METHODS

Organisms

Twenty-seven yeasts were the generous gift of Dr. S.A. Crow, Department of Biology, Georgia State University, Atlanta, GA. Their strain numbers and their original sources are shown in Table 1. Two additional yeasts, *Saccharymyces cerevisiae* X2180–1B and *Zygosaccharomyces rouxii* NCYC 1522 were from the collection of GMG. *A. pullulans*, a member of the Fungi Imperfecti with a yeast-like stage, was also included in the study.

The organisms were maintained on Sabouraud's dextrose or MYGP agar slants. Inocula were taken from 24 to 48-h cultures on the surface of plates of MYGP medium which contained ($g \cdot l^{-1}$ of double-distilled water): malt extract (Lab M) 3, yeast extract (Lab M) 3, bacteriological peptone (Oxoid) 5, D-glucose 10 and Agar (Lab M, No. 2) 12. Cells were suspended in sterile distilled water to an optical density of approximately 1.0 at 550 nm.

Organometals

Two organoleads and nine organotins were used. $(CH_3)_2PbCl_2$ and $(CH_3CH_2)_2PbCl_2$ were the gift of Dr. Y.K. Chau of the Canada Centre for Inland Waters, Burlington, Ontario. CH_3SnCl_3, $(CH_3)_2SnCl_2$, $(CH_3)_3SnCl$, and mono-, di- and tri-*n*-butyltin chlorides were from Alfa Products, Danvers, MA. $(C_2H_5)_2SnCl_2$ and $(C_2H_5)_3SnBr$ were from Aldrich Chemical Co. (Milwaukee, WI). Triphenyltin chloride was from Fluka AG, Buchs, Switzerland. Stock solutions of organometals were prepared in absolute ethanol.

Table 1

Origins of the twenty-nine yeasts used in the screening studies

Organism	Strain no.	Isolated from
Aureobasidium pullulans	NS-76-122	North Sea
Candida albicans	NS-75-33	North Sea
Candida albicans	NS-75-45	North Sea
Candida albicans	NS-75-52	North Sea
Candida albicans	NS-75-56	North Sea
Candida albicans	NS-75-57	North Sea
Candida albicans	NS-75-59	North Sea
Candida albicans	CA-30	Clinical isolate
Candida boidinii	NRRL 2332	Industrial fermentation
Candida guillermondii	NS-76-148	North Sea
Candida guillermondii	NS-76-299	North Sea
Candida lipolytica	37-1	Frankfurter
Candida maltosa	CBS-6680	Soil
Candida maltosa	CBS-5611	Monsodium glutamate neutralizing tank
Candida parapsilosis	NS-76-146	North Sea
Candida pulcherrima	NS-76-147	North Sea
Candida tropicalis	32113	Hydrocarbon-contaminated soil
Debaryomyces hansenii	NS-75-11	North Sea
Debaryomyces hansenii	NS-75-21	North Sea
Hanseniaspora sp.	NS-76-136	North Sea
Pichia anomala		Lake Champlain
Pichia pinus		Forest soil
Rhodotorula rubra	NS-76-138	North Sea
Rhodotorula rubra	NS-76-179	North Sea
Saccharomyces cerevisiae	8112	Industrial fermentation
Saccharomyces cerevisiae	X2180-1B	
Sporobolomyces albo-rubescens	NS-76-199	North Sea
Torulopsis candida	NS-76-300	North Sea
Zygosaccharomyces rouxii	NCYC 1522	

Methods for screening

Center well diffusion plates were prepared using a defined agar (DA) medium which contained (g ·

l^{-1}): glucose 10, yeast N-base (Difco) 6.7 and purified agar (Oxoid) 12. Each 90-mm diameter Petri dish received 10 ml of medium. Each plate was spread-inoculated with 0.1 ml of cell suspension and a 6-mm diameter well was then cut aseptically in the plate center; a 50-μl aliquot of organometal solution or absolute ethanol, as a control, was added to the well. Plates were incubated at 25°C for 40–48 h in the dark to avoid any light-induced decomposition of the organometals. The zones of inhibition were then measured.

For density gradient plates, 12 ml MYGP agar, containing the desired concentration of organometallic compound (added to the molten agar at 50°C) was layered over 12 ml organometal-free MYGP agar in a 90-mm diameter Petri dish in a modification of the method of Szybalski and Bryson [25] as described by Newby and Gadd [22]. Plates were spread-inoculated and incubated as above. Growth was quantified by removing eight cores of agar (6-mm diameter) equally spaced along the organometal concentration gradient. Each core was agitated using a vortex mixer in 5 ml H_2O and the optical density of the resulting suspension was measured at 550 nm.

Two-dimensional gradient plates [29] were used to estimate the effect of pH on organometal toxicity as described by Newby and Gadd [22]. Each 10 × 10 cm square plate received 60 ml of DA medium. Wells (1 × 7 cm) were cut aseptically in the agar on two adjacent sides, and 1 ml of the desired organometal solution was placed in one well and 2 ml of 0.1 M HCl, 0.1 M KOH or 1 M KOH was placed in the other well. After 24 h incubation at 25°C in the dark, diffusion was stopped by inserting a sterile stainless steel grid, containing 49 1-cm compartments, into the agar. Each compartment was inoculated with a loop of cell suspension. Plates were incubated for 48 h at 25°C in the dark and then each compartment was scored for growth. The pH gradient on the plate was determined using a Pye-Unicam 403-30-M3 combination surface electrode and a Kent EIL model 7055 pH meter.

Release of K^+ from cell suspensions was measured using a K^+-electrode. Cells were grown in a liquid medium of composition ($g \cdot l^{-1}$): glucose 20, yeast extract (Difco) 1, KH_2PO_4 2.72, K_2HPO_4 5.22, $(NH_4)_2SO_4$ 1.98, $MgSO_4 \cdot 7H_2O$ 0.12, $MnSO_4 \cdot 4H_2O$ 0.004, $FeSO_4 \cdot 7H_2O$ 0.0022, $ZnSO_4 \cdot 7H_2O$ 0.004 and $CuSO_4 \cdot 5H_2O$ 0.0004 for 18 h (mid-exponential phase). Cells were harvested by centrifugation at 1500 × g for 5 min, washed three times and finally resuspended to a density of approximately 10^7 cells ml^{-1} in 20 ml 5 mM piperazine-*N*,*N*′-bis[2-ethane sulphonic acid] (PIPES) buffer, adjusted to pH 6.5 using tetramethylammonium hydroxide. Cell suspensions were incubated at 25°C for 15 min on a magnetic stirrer and glucose was added to a final concentration of 50 mM. Efflux of K^+ was continuously recorded using a potassium-specific electrode (Corning) in conjunction with a Kent/EIL model 7055 pH meter connected to a Servoscribe potentiometric chart recorder.

RESULTS AND DISCUSSION

Results of center well diffusion assays are shown in Table 2. Throughout the paper typical results are presented, although each experiment was done in duplicate or triplicate. Tributyltin was the most toxic compound tested, followed by triphenyltin and triethyltin. In general, tri-substituted compounds were more toxic than mono- or disubstituted organotins but there were different patterns of sensitivity depending on the organism. For example, several strains were more sensitive to mono- and/or dimethyltins than to trimethyltin, and tributyltin was not always the most toxic butylated tin compound. Dimethyllead was more toxic than diethyllead, probably due to differences in diffusion, and not all organisms which were sensitive to dimethyltin were sensitive to dimethyllead. Center well diffusion plates produced small zones of inhibition even at the highest concentrations of organometals used. Therefore, this method is not useful for comparing compounds where there are differences in their diffusion rates, but the method has value for comparing the sensitivities of different organisms to a single organometal, particularly for the most toxic compounds.

Typical results from Szybalski density gradient

Table 2

Toxicity of nine organotins and two organoleads to twenty-nine yeasts determined using center well diffusion plates[a]

Yeast	Diameters of zones of inhibition (mm)			
	$MeSnCl_3$ (10^{-2} M)	Me_2SnCl_2 (10^{-2} M)	Me_3SnCl (10^{-2} M)	Et_2SnCl_2 (10^{-3} M)
A. pullulans	20	0	18	0
C. albicans NS-75-33	0	0	0	0
C. albicans NS-75-45	0	15	0	0
C. albicans NS-75-52	0	0	0	0
C. albicans NS-75-56	0	17	0	0
C. albicans NS-75-57	0	0	0	0
C. albicans NS-75-59	0	0	0	0
C. albicans Ca-30	0	0	0	0
C. boidinii	0	0	0	0
C. guillermondii NS-76-148	19	16	0	0
C. guillermondii NS-76-299	0	0	17	0
C. lipolytica	0	17	0	0
C. maltosa CBS-6680	0	0	0	0
C. maltosa CBS-5611	0	0	15	0
C. parapsilosis	0	18	0	0
C. pulcherrima	17	19	0	0
C. tropicalis	0	0	17	0
D. hansenii NS-75-11	0	0	30	3
D. hansenii NS-75-21	14	0	0	5
Hanseniaspora sp.	0	0	18	0
P. anomala	0	0	0	0
P. pinus	18	17	0	0
R. rubra NS-76-138	0	0	25	0
R. rubra NS-76-179	0	0	25	0
S. cerevisiae 8112	0	18	13	0
S. cerevisiae X2180-1B	20	0	0	0
S. albo-rubescens NS-76-199	16	0	20	18
T. candida	15	0	0	0
Z. rouxii	0	0	14	0

[a] Each plate received 50 µl of a solution of organotin in ethanol at the concentration shown above each column. Zones of inhibition have been corrected for well diameter and an ethanol control (generally negligible).
The notations used indicate: $MeSnCl_3$, Me_2SnCl_2 and Me_3SnCl = mono-, di- and trimethyltin chlorides, respectively; Et_2SnCl_2 = diethyltin chloride; Et_3SnBr = triethyltin bromide; $BuSnCl_3$, Bu_2SnCl_2 and Bu_3SnCl = mono-, di- and tributyltin chlorides; triphenyl SnCl = triphenyltin chloride; Me_2PbCl_2 and Et_2PbCl_2 = dimethyl- and diethyllead chlorides, respectively.

plates are shown in Fig. 1. It was assumed that diffusion of the organometal into the lower layer was proportional to the thickness of the plate and that this resulted in an approximately linear gradient of organometal across the plate as previously shown for inorganic metal compounds [13] and antibiotics [25]. The half-inhibitory concentration (HIC) can be estimated from the plot. A summary of half-inhibitory concentrations for ten yeasts is shown in Table 3. According to this method, tributyltin was the most toxic compound. Tri-substituted compounds, with the exception of triphenyltin, were more toxic than their mono-or di-substituted homologues. For triphenyltin, the response varied and

Et_3SnBr		$BuSnCl_3$	Bu_2SnCl_2	Bu_3SnCl	Triphenyl SnCl		Me_2PbCl_2	Et_2PbCl_2
(10^{-3} M)	(10^{-2} M)	(10^{-2} M)	(10^{-2} M)	(10^{-3} M)	(10^{-3} M)	(10^{-2} M)	(10^{-2} M)	(10^{-3} M)
5	26	0	20	6	7	19	0	0
11	29	0	19	20	11	19	0	0
10	25	0	0	17	9	13	0	0
5	20	0	0	12	8	14	0	0
0	27	0	17	17	8	12	0	0
7	23	0	0	24	8	9	0	0
5	16	0	0	24	9	15	0	0
0	19	0	0	26	9	13	0	0
0	3	0	0	17	0	7	0	0
9	36	0	0	28	3	12	21	0
0	18	0	0	23	0	0	0	0
4	13	0	0	13	5	7	15	0
0	4	0	0	7	0	0	19	0
4	5	20	0	7	0	7	0	0
0	5	20	0	2	0	5	0	0
10	18	15	18	15	5	10	0	0
0	5	0	0	11	0	8	0	0
11	26	0	0	16	3	12	23	4
14	32	0	20	20	2	15	0	4
0	14	0	0	23	0	4	0	0
0	19	0	0	18	0	19	0	0
0	11	0	0	14	0	7	18	0
19	34	0	0	12	7	13	26	2
20	34	0	0	18	8	8	0	0
0	6	17	0	14	0	11	0	0
0	15	17	0	12	7	24	0	0
19	33	0	0	12	14	23	0	0
0	24	0	0	15	0	5	16	0
0	16	0	0	20	7	13	0	0

ranged from complete inhibition to relative insensitivity. As with center well diffusion plates there were individual patterns of sensitivity among the ten yeasts examined. This method provides quantitative information on toxicity and allows comparisons between organisms, but it is also limited by differences in diffusion rates of the organometals.

Results of two-dimensional gradient plates are shown in Fig. 2. For both experimental organisms, toxicity was assessed over a range of pH from 5.5–8.4 although for clarity, only selected data are presented. Tributyltin was more toxic to *A. pullulans* at acid pH than at neutral pH. However, it was less toxic to *R. rubra* at pH 7.7 or 7.9 than at higher or

Table 3

Toxicity of nine organotins and two organoleads to ten yeasts using density-gradient plates[a]

	Half-inhibitory concentration (μM) of:										
	$MeSnCl_3$	Me_2SnCl_2	Me_3SnCl	Et_2SnCl_2	Et_3SnBr	$BuSnCl_3$	Bu_2SnCl_2	Bu_3SnCl	Tryphenyl SnCl	Me_2PbCl_2	Et_2PbCl_2
A. pullulans	18	14	16	66	44	18	27	10	51	18	24
C. albicans CA30	30	32	20	39	ng	18	12	ng	10	34	36
D. hansenii 75-11	24	36	20	12	ng	30	30	ng	38	32	28
D. hansenii 75-21	52	ni	36	52	ng	60	48	ng	35	20	60
P. anomala	58	48	24	36	ng	32	33	ng	ng	36	24
P. pinus	50	42	40	54	40	42	41	ng	25	34	46
R. rubra NS-76-138	20	30	ng	30	ng	24	24	ng	75	18	16
R. rubra NS-76-179	18	38	22	19	ng	24	16	ng	51	12	ng
S. cereviseae X2180-1B	ni	38	28	25	ng	ni	72	ng	25	24	40
S. albo-rubescens	2	24	ng	11	ng	18	14	ng	52	ng	ng

Notations as in Table 2. ng = no growth on plate at the lowest concentration used; ni = no inhibition of growth over the concentration range used. Organometal concentrations as in Fig. 1.

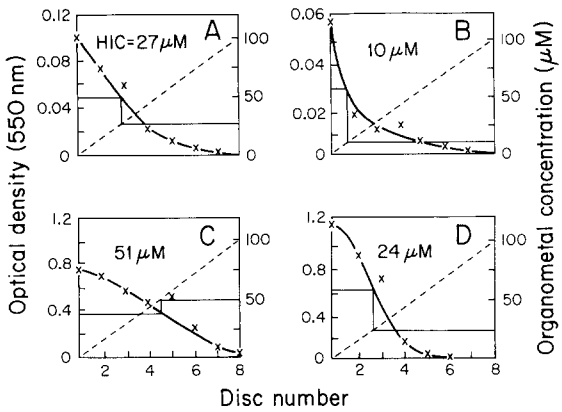

Fig. 1. Toxicity of organometals to *Aureobasidium pullulans* NS-76-122 assayed by density gradient plates. Disc 1 corresponds to the highest organometal concentration. The half-inhibitory concentration (HIC) is shown on each plot. ×——× optical density at 550 nm of cell suspension washed from agar disc, – – – – – concentration of organometal, the gradient is assumed to be linear across the plate. A, 10^{-4} M tributyltin chloride in the top layer of agar; B, 10^{-4} M dibutyltin dichloride; C, 10^{-4} M triphenyltin chloride; D, 10^{-4} M diethyllead dichloride.

lower pH values. Thus, effects of pH on the toxicity of individual organometals are readily demonstrated using this method but, as with the other methods, differences in diffusion rates may limit it for comparisons of toxicity between compounds.

Organometal-induced release of K^+ from cells of *A. pullulans* is shown in Fig. 3. Mono- and tributyltins were the most toxic of the 11 organometals tested against *A. pullulans*. The relative toxicities of the organometals as assessed by K^+ release differed from the toxicities measured by the agar diffusion methods (Tables 2 and 3) which emphasizes likely differences in agar diffusion rates among the organometals. The toxicities of three butyltins and triphenyltin to four yeasts and *A. pullulans* are summarized in Fig. 4. Differences in sensitivity patterns are evident between the organisms. *Z rouxii* was the most sensitive to all four compounds whereas *A. pullulans* was sensitive to mono- and tributyltins but not to dibutyltin or triphenyltin. Of the five organisms *A. pullulans* was the most sensitive to monobutyltin and the least sensitive to triphenyltin. *P. pinus* was sensitive to all compounds except dibutyltin. *S. cerevisiae* was not sensitive to dibutyltin but released K^+ in the presence of the other three compounds.

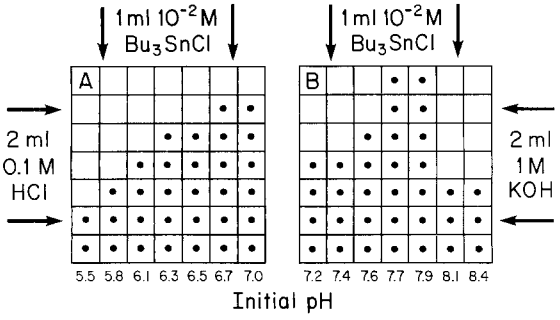

Fig. 2. Diagram of results obtained with two-dimensional diffusion plates. Tributyltin was allowed to diffuse in one direction for 24 h while an acid or base was diffusing at a right angle to it. A stainless steel grid was then pressed into the agar to prevent further diffusion and the medium in each compartment was inoculated. A dot in the compartment indicates that growth occurred. A, *A. pullulans;* B, *R. rubra* NS-76-138.

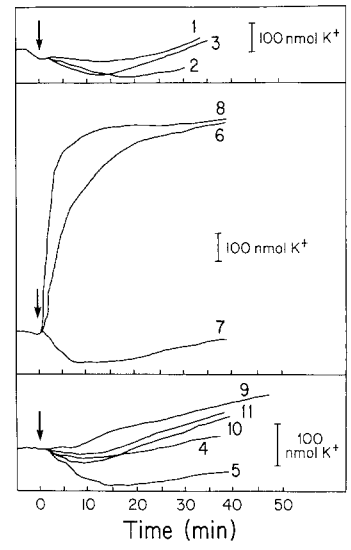

Fig. 3. Organometal-induced release of K^+ from cells of *A. pullulans*. Arrows indicate the time at which the organometal solution was added. Final concentrations in the cell suspension were 50 μmolar except for trimethyltin chloride which was used at 45 μmolar, and diethyltin dichloride and diethyllead dichloride which were used at 5 μmolar. 1, $MeSnCl_3$; 2, Me_2SnCl_2; 3, Me_3SnCl; 4, Et_2SnCl_2; 5, Et_3SnBr; 6, $BuSnCl_3$; 7, Bu_2SnCl_2; 8, Bu_3SnCl; 9, triphenyltin chloride; 10, Me_2PbCl_2; 11, Et_2PbCl_2.

High intracellular concentrations of K^+ are found in fungi and measurement of K^+ efflux has been used to assess the toxicity of antibiotics, xeno-

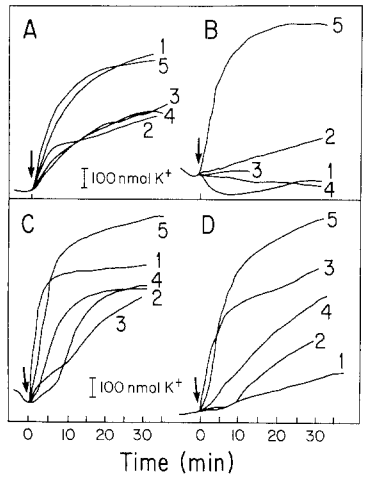

Fig. 4. Organotin-induced release of K^+ from five yeasts. A, monobutyltin trichloride; B, dibutyltin dichloride; C, tributyltin chloride; D, triphenyltin chloride. Final concentrations in the cell suspension were 50 μmolar. 1, *A. pullulans*; 2, *D. hansenii* NS-75-21; 3, *P. pinus*; 4, *S. cerevisiae* X2180-1B; 5, *Z. rouxii*. Organometal solution was added where indicated by the arrow.

biotics and other heavy metals [11]. Regard should be given to potential complicating factors since, in some cases, K^+ efflux and toxicity may be unconnected or may not show a direct relationship [3,26]. Nevertheless, this method appears satisfactory for assessment of comparative organotin toxicity, particularly in view of the fact that the toxic action of organotins is mediated by interaction with the cell membrane [7,8].

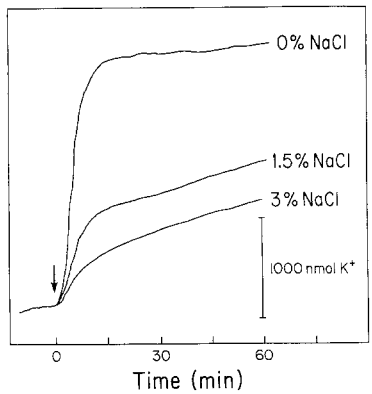

Fig. 5. Effect of NaCl on K^+ release from *Z. rouxii* induced by tributyltin chloride which was added at the time indicated by the arrow to a final concentration of 50 μM.

The effect of 1.5 and 3% (wt/vol) NaCl (approximating estuarine and seawater conditions, respectively) on tributyltin-induced K^+ loss from *Z. rouxii*, previously grown in the absence of NaCl, is shown in Fig. 5. Both the rate and extent of K^+ release was decreased with increasing salinity indicating some modification of organotin toxicity. NaCl alone did not cause any significant K^+ release. While Na^+ can wholly or partially substitute for K^+ in Na^+-grown *S. cerevisiae* [2], preliminary experimentation confirmed that the reduced K^+ release did not reflect a reduced level of intracellular K^+ (results not shown). It is possible that Na^+ prevents uptake of the tributyltin moiety and thus prevents toxicity either by interaction with the cells or with the compound itself. Alternatively, it has been shown that the presence of Cl^- in seawater can inhibit the solubility of tributyltin compounds by association with the cation to form covalent organotin chloride [1]. It should be also borne in mind that transfer of *Z. rouxii* from NaCl-free medium to one containing 2 M NaCl can affect the membrane lipid composition [28] which could alter the interaction of organometals with the cell membrane. It is clear that such effects may be highly significant in relation to the efficacy of organotins as components of antifouling paints and to their toxicity in the marine environment [5].

Of the organometals tested in this study, tributyltin was the most toxic, and mono- and dimethyltins were the least toxic. Among the eight compounds which showed intermediate toxicity, triphenyltin and monobutyltin were the most toxic. These results are consistent with the concepts that trisubstituted organotins are generally more toxic than mono-, di- or tetrasubstituted tins, and that of trisubstituted tins those with 9 to 15 total carbons in organic substituents are the most toxic to microorganisms [7,17,20,27,30].

A variety of chemical and physical conditions can influence the toxicity of organometals to microorganisms including the growth stage of the organisms, pH and the presence of surface-active agents [30] as well as organic and inorganic components of the medium and even the solidifying agent used to prepare plates [10,12,16,19]. Thus, caution must be

used when extrapolating toxicity data from laboratory to in situ situations, and when comparing the results obtained by two different toxicity assay methods.

Among the four methods examined in the present work, center well diffusion assays appear to be limited to comparison of sensitivities among different organisms to single organometals of high toxicity. Density gradient plates are also limited for comparing compounds which diffuse at different rates but they provide quantitative data and they require no special equipment. Two-dimensional gradient plates are limited by diffusion rates but if high concentrations of organotins are used they can be useful for screening for effects of pH, salt and other diffusible chemicals on toxicity [11]. Release of K^+ from cells does not suffer from the limitations imposed by diffusion through a gel, it is rapid and provides for manipulation of a large number of environmental variables.

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