Environmental influences on organotin-yeast interactions

Ph.D. Research Thesis by

Jane S. White B.Sc.

Supervisor

Dr. John M. Tobin

School of Biotechnology

Dublin City University

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: Jane Wilde

ID No: 96970260

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"The great tragedy of Science – the slaying of a beautiful hypothesis by an ugly fact"

TH Huxley (Collected Essays, 1893-94)

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ABSTRACT

As a consequence of the widespread industrial and agricultural applications of organotin compounds, contamination of various ecosystems has occurred in recent decades. Understanding how these compounds interact with cellular membranes is essential in assessing the risks of organotin pollution. The organotins, tributyltin (TBT) and trimethyltin (TMT) and inorganic tin, Sn(IV), were investigated for their physical interactions with non-metabolising cells and protoplasts of the yeast, *Candida maltosa*, an organism that is often associated with contaminated environments. Sn(IV) and TBT uptake occurred by different mechanisms. TBT uptake resulted in cell death and extensive K^+ leakage, while Sn(IV) uptake had no effect. TMT did not interact with cells. Of the three compounds, TBT alone altered the membrane fluidity of cells, as measured by the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene incorporated into cells. To further examine the contribution of lipophilic interactions, the influence of pH and NaCl concentration on TBT and triphenyltin (TPT) uptake and toxicity was assessed. Solution pH and ionic composition influence the chemical speciation and toxicity of organotins in the aquatic environment. Organotin compounds may exist as both hydrated cationic species and neutral hydroxides in solution, with the formation of chloride species in the presence of NaCl. The uptake and toxicity of TBT and TPT by *C. maltosa* was investigated between pH 3.5 and 7.5 and in concentrations of up to 500 mM NaCl. A theoretical model was used to predict the speciation and overall octanol-water distribution ratios (D_{ow}). TBT and TPT toxicity was correlated with D_{ow} values, corresponding to increasing pH and NaCl concentration and implicating compound lipophilicity as a toxicity determinant.

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PUBLICATIONS

White, J.S., Tobin, J.M. and Cooney, J.J. (1999) Organotin compounds and their interactions with microorganisms. Can. J. Microbiol. **45**: 541-554.

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CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

Organotin compounds comprise organometallic moieties characterised by a Sn atom covalently bound to one or more organic substituents. Chemically, these compounds are represented by the formula $RSnX_3$, R_2SnX_2, R_3SnX and R_4Sn, where R is an alkyl or aryl group and X is an anionic species such as halide, oxide or hydroxide. The toxic effect of organotins has been recognised as far back as 1954, when an attempt to use organotin compounds for treatment of staphylococcal infections resulted in the death of 102 people (Zuckerman et al., 1978). Their industrial applications and use as general biocides have resulted in widespread environmental pollution. The following sections aim to provide a comprehensive review of the interactions of organotins with microorganisms and to introduce the experimental objectives of this work.

1.1 Organotin concentrations in the environment

Organotin compounds are ubiquitous in the environment and have a wide range of industrial and agricultural applications. Organotins have been detected in water, sediments and biota of both freshwater and estuarine environments (Fent and Hunn 1991; Schebeck et al., 1991; Weurtz et al., 1991), as a contaminant of wastewater and sewage sludge (Fent and Müller, 1991) and have also been detected in soil as a result of dumping of sewage sludge or from use as agrochemicals (Fent 1996a). The introduction of organotins into the environment and their concentrations in ecosystems are discussed below.

1.1.1 *Organotin applications and entry into the environment*

Organotins have diverse industrial and biocidal applications (Table 1.1) and tin may have more of its organometallic derivatives in use than any other element (Blunden et al., 1984). Monoalkyltins and dialkyltins are mainly utilised for nonbiological purposes with the major application (approximately 70% of the total organotin use) as heat and light stabiliser additives in PVC processing (Hoch 2001).

Table 1.1 Applications of organotin compounds[a]

Applications		Compounds[b]
Non-biological uses:	Thermal and UV-stabilisers for rigid and semi-rigid PVC	$RSn(SCH_2.CO.OOct^i)_3$[c] (R = Me,Bu,Oct)
		$(BuSnS_{1.5})_4$
		$R_2Sn(SCH_2.CO.OOct^i)_2$ (R = Me,Bu,Oct)
		$(R_2Sn(O.CO.CH{:}CH.CO.O)_n$ (R = Bu,Oct)
		$R_2Sn(O.CO.CH{:}CH.CO.OR')_2$ (R = Bu, R' = Oct)
	Catalysts for production of polyurethane foams, silicones and transesterification reactions	$(Bu_2SnO)_2$
		$\{Bu_2Sn(O)OH\}_n$
		$BuSn(OH)_2Cl$
	Precursor for forming SnO_2 films on glass	$BuSnCl_3$
		$MeSnCl_3$ (20%) + Me_2SnCl_2 (80%)
		Me_2SnCl_2
Biological uses:	Agrochemicals :	
	Fungicides	Ph_3SnX (X=OH, OAc)
	Acaricides	$(CyHex)_3SnOH$
		$\{(PhMe_2CCH_2)_3Sn\}_2O$
	Antifeedants	Ph_3SnX (X=OH,OAc)

Applications	Compounds
Antifouling paint biocides	Ph_3SnX (X=OH,OAc, F, Cl, S.CS.NMe_2)
	$Ph_3SnO.CO.CH_2CBr_2.CO.$
	$OSnPh_3$
	Bu_3SnX (X=F,Cl,OAc)
	$(Bu_3Sn)_2O$
Wood preservative fungicides	$(Bu_3Sn)_2O$
	Bu_3Sn(naphthenate)
	$(Bu_3Sn)_3PO_4$
Stone preservation	$(Bu_3Sn)_2O$
Disinfectants	$Bu_3SnO.CO.Ph$
	$(Bu_3Sn)_2O$
Molluscicides	Bu_3SnF
	$(Bu_3Sn)_2O$
Anthelmintics for poultry feeds	$Bu_2Sn(O.CO.C_{11}H_{23})_2$

[a] Compiled from Blunden et al. (1984) and Cooney and Wuertz (1989).

[b] Notations used throughout include: Me = Methyl, Et = Ethyl, Pr = Propyl, Bu = Butyl, Ph = Phenyl and CyHex = cyclohex, Ac = acetate.

[c] These compounds are used in combination with the corresponding R_2SnX_2 derivatives.

Triorganotins are exploited for their fungicidal, bactericidal, algicidal and acaricidal properties with approximately 23% of the total worldwide organotin production used as agrochemicals and as general biocides (Fent 1996b). A few divalent organotins also exist but they are insignificant because they have no practical use (Hoch 2001)

Tributyltin (TBT) pollution has received the most attention and is related to its use in antifouling paints. Toxicity towards non-target organisms has led to legislation restricting its use. Growing production of tributyltin is also related to its use as preservative for timber, wood, textiles, paper and leather (Fent, 1996b). Since the early 1960s, triphenyltin (fentin) hydroxide and triphenyltin acetate have been used in agriculture, where they are used as fungicides to protect crops, including potato, celery, sugar beet, and rice and to prevent tropical diseases in peanuts, pecans, coffee and cocoa (Hoch 2001). Triphenyltin (TPT) has also been employed as a cotoxicant with TBT in some antifouling paints (Fent and Hunn, 1991).

The possible routes of entry and dispersal of organotins in the environment are summarised in Fig 1.1. Although the agricultural and biocidal uses of organotins comprise only a fraction of the total consumption, they give rise to the largest proportion of free organotins in the environment, due to direct introduction into soil, air and water (Blunden et al., 1984). TBT compounds are directly introduced into aquatic systems via leaching from antifouling paints and this route is still of paramount importance. Organotins also enter the environment as a result of agricultural applications, where run-off and leaching from soil can lead to contamination of nearby rivers and streams (Fent and Hunn 1991). Leaching and normal weathering of PVC products can lead to inputs of methyltins and butyltins into the aquatic and terrestrial environment (Fent 1996a). Other less important routes of entry result from disposal of materials manufactured with organotins in landfills, from treated surfaces of preserved materials or air emissions from municipal waste incineration or during application of agrochemicals (Fent 1996a).

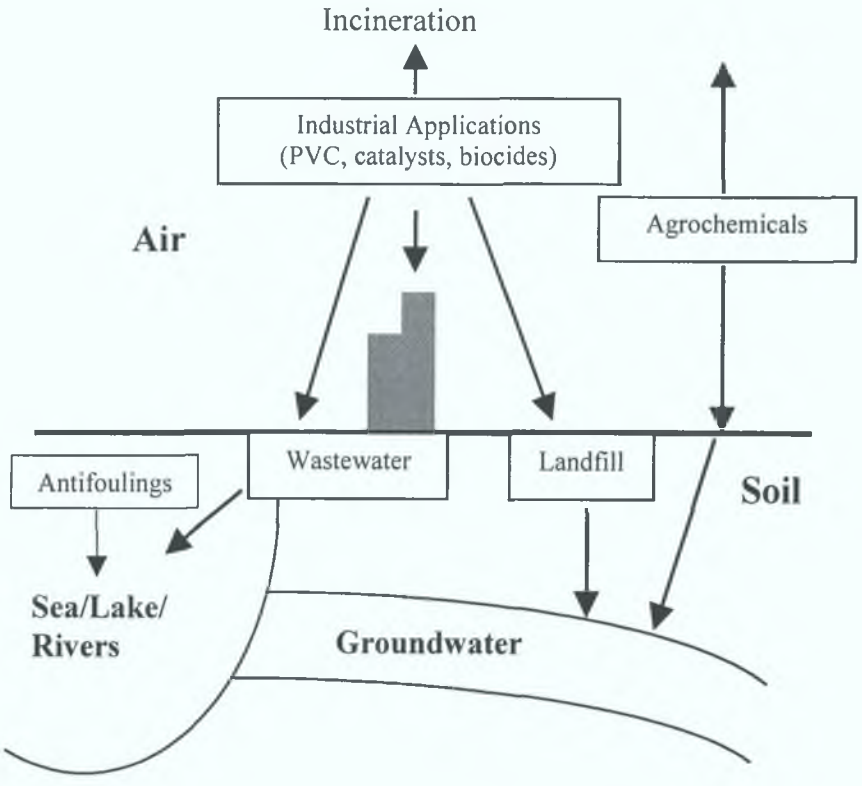

Figure 1.1 Organotin entry routes into the environment (modified from Fent 1996a.)

1.1.2 *Tributyltin legislation*

Research on tributyltin has mainly focused on its use in antifouling paints, which are used to coat structures exposed to an aquatic environment, including ships, pleasure boats, buoys, pilings, sea walls, oil rigs, cables and water intake pipes (Cooney et al., 1994). The use of antifouling paints on ships prevents the build-up of nuisance organisms, which results in greater hull friction and increased fuel usage and reduces the spread of invasive (non-native) species across the world's oceans. The biocide is released from the paint with time, resulting in the formation of a thin layer of concentrated TBT in the immediate vicinity of the painted surface, which repels or kills nuisance organisms such as barnacles (Huggett et al., 1992). However, TBT can diffuse further, leading to contamination of adjacent water and sediments and exposure to

nontarget organisms. The toxic effects of TBT on nontarget organisms have led to restrictions on its use in antifouling paints.

France was the first country to implement restrictions when, in 1982 the use of TBT-containing paints on boats shorter than 25m in length was banned (Huggett et al., 1992). The reasoning behind this was that smaller boats spend more time in harbours and closer to the shore, where TBT is more likely to accumulate in sediments, while larger vessels spend the majority of their time at sea resulting in dilution of the biocide in the greater volume of water. Aluminium boats were exempted, as the alternative copper-based paints would result in severe corrosion. Britain was next to introduce legislation in 1985, which was aimed at controlling the release rate of TBT from paints. In the United States the "Organotin Antifouling Paint Control Act of 1988" regulates the use of TBT. This restricts painting of vessels shorter than 25m (aluminium hulls exempted) and required paints to leach at a rate of no greater than 4.0μg/cm^2/day (Huggett at al, 1992).

TBT can be toxic at extremely low concentrations, with molluscs, bivalves and gastropods as the most sensitive species (Table 1.2). As a result, the International Convention on the control of harmful antifoulant systems on ships was adopted by the International Maritime Organisation in October 2001. Under the terms of the convention the use of TBT-based antifoulants will be banned by January 2003 with a complete prohibition on the presence of the chemical in marine paints by 1st January 2008.

Table 1.2 Summary of the toxic effects of TBT on marine organisms (adapted from Alzieu, 1998)

Concentration of TBT	**Effect observed**
Less than 1 ng L^{-1}	Appearance of imposex in gastropods
Exceeding 1 ng L^{-1}	Limit of cell division in phytoplankton and reproduction of zooplankton
Exceeding 2 ng L^{-1}	Shell calcification anomalies in the oyster *Crassostrea gigas*
Exceeding 20 ng L^{-1}	Disturbances in the reproduction of bivalve molluscs
1-10 μg L^{-1}	Effects on fish reproduction
1-1000 μg L^{-1}	Disturbed fish behaviour

1.1.3 *Organotin pollution of aquatic systems*

Detection of organotins in aquatic systems has mainly focused on TBT and its degradation products, dibutyltin (DBT) and monobutyltin (MBT). Evidence exists to indicate that legislation governing TBT use in antifouling paints has led to a reduction in organotins in the aquatic environment. There have been several reports on declining levels of TBT in coastal waters (Evans et al., 1995). Tin tissue burdens have declined in oysters and mussels, there has been substantial improvement in the health of oyster populations and oyster farming has recovered (Evans et al., 1995). In Lake Lucerne, Switzerland, TBT concentrations in freshwater boat harbours declined from 470 ± 190 ng L^{-1} in 1988 to 50 ± 40 ng L^{-1} in 1993 (Fent and Hunn, 1995). Between this period TPT was detected at levels up to 50 ng L^{-1}. Distribution of organotins in UK rivers has also declined (Dowson et al., 1994). Of 12 freshwater sites in the Norfolk Broads, UK, which had previously been found to be contaminated with TBT only one site had detectable butyltin concentrations in the water column, at 13 ng L^{-1}.

Based on this evidence, it has been argued that a total ban on TBT is premature and should not be enforced until an environmentally and economically sound alternative is developed (Evans 1999, Evans et al., 2000). However, recent surveys indicate that concentrations still exceed the environmental quality standard value of 2 ng L^{-1}, set by the UK (Fent, 1996b). TBT pollution is also still a problem in French waters, where the initial reduction in concentrations after the introduction of legislation has ceased (Michel and Averty, 1999). Of 237 measurement points sampled, 75% of the dissolved TBT concentrations were above the threshold of 1 ng L^{-1}, while 25% of measurements exceeded 21.4 ng L^{-1}. In countries where no regulations have been implemented, levels are in the same range as other countries prior to legislation (Fent, 1996b). This is evident in Asia, Africa and South America.

In contrast to the apparent reduction in organotin concentrations in the water column, organotins often persist at high concentrations in sediments. As degradation rates are in the order of years (Astruc et al., 1989; De Mora et al., 1989; Dowson et al., 1993b), sediments remain contaminated for longer time periods. In the top layer sediment from Lake Lucerne, Switzerland, TBT and TPT were found at concentrations of 2.8 and 0.38 μg (g dry wt.)$^{-1}$, respectively, with very little degradation products

detected (Fent and Hunn, 1995). Between 1990 and 1993, TBT concentrations in sediments from Lake Geneva remained between 1.56 and 2.47 μg (g dry wt.)$^{-1}$ (Becker-van Slooten and Tarradellas, 1995). In contrast, concentrations in surficial sediments from freshwater sites (East Anglia, UK) had reduced since the introduction of legislation (Dowson et al., 1994). TBT detected in the range 0.01-1.3 μg Sn (g dry wt.)$^{-1}$ in 1987, was reduced to <0.003-0.069 μg Sn (g dry wt.)$^{-1}$.

Distribution of organotins in the water and sediment layers may determine bioavailability and toxicity to organisms. In freshwater, MBT and to a lesser extent TBT, is partitioned towards the particulate phase, while DBT exhibits an approximate 50:50 partitioning between particulate and solution phases (Dowson et al., 1993a). In estuarine waters, MBT will almost exclusively be absorbed onto particulates, TBT will be predominantly in the solid-phase fractions, with 10-30% remaining in solution, while DBT is solubilised. TBT sorption is reversible (Dowson et al., 1993a), indicating that contaminated sediments may release TBT. Mechanical operations such as dredging and natural disturbances such as storms or bioturbation may facilitate the release of TBT into the overlying water column.

The lipophilic nature conferred to organotins by the alkyl groups facilitates their bioaccumulation by living organisms. Of growing concern is the possibility of the compounds being concentrated through the food chain as a result of bioaccumulation. Bioconcentration factors (BCF) may range between 100 – 500 000 for microorganisms, macroalgae, invertebrates, fish, birds and mammals (Laughlin, 1996). Organotins still occur at elevated levels in higher organisms, even after legislation (Fent, 1996b). Of 38 species of Japanese gastropods surveyed, 30 species were affected by imposex (Horiguchi et al., 1995). Occurrence rates in rock shells, *Thais clavigera* and *T. bronni* were 100% at almost all sites surveyed and were positively related to both TBT and TPT concentrations. Butyltin residues have also been detected in marine mammals. Hepatic butyltin concentrations in finless porpoise from the Seto Island Sea, Japan as high as 10 000 ng (g wet wt.)$^{-1}$ have been reported, while other cetaceans from the Japanese coastline had butyltin concentrations ranging from 110 - 5 200 ng (g wet wt.)$^{-1}$ (Tanabe et al., 1998).

1.1.4 *Organotin pollution of terrestrial environments*

Reports of organotins in environments other than aquatic systems are scarce. Effluent from municipal water treatment systems, released into the Rhine river was found to contain inorganic tin, dimethyltin (DMT), MBT and DBT, in the concentration ranges 153 - 200, 0.8 -2, 9 and 5 - 8 ng Sn L^{-1}, respectively (Schebeck et al., 1991). Input into the municipal plants likely results from direct industrial use of these compounds or from the industrial or private use of articles containing tin, for example PVC drain pipes.

In a wastewater treatment system organotins become primarily associated with the particulate phase and are removed mainly by sedimentation in the primary clarifier. In a Swiss municipal wastewater treatment plant organotins were detected at levels ranging from 136 - 564 ng MBT L^{-1}, 127 - 1026 ng DBT L^{-1}, and 64 - 217 ng TBT L^{-1} (Fent and Müller, 1991). After primary sedimentation, 73% of the total organotins had been removed, while levels in digested sludge were 0.78 ± 0.19 mg (kg dry wt.)$^{-1}$ MBT, 0.98 ± 0.3 mg (kg dry wt.)$^{-1}$ DBT and 0.99 ± 0.91 mg (kg dry wt.)$^{-1}$ TBT. As organotins become concentrated in sludge, the risks associated with disposal need to be assessed.

Despite the use of TPT in agrochemicals, there is very little information concerning concentrations in soils. Total phenyltin concentrations (mono-, di-, and triphenyltin) in foliage and soils of pecan orchards immediately after application of TPTOH was 72 and 26 μg Sn (g dry wt)$^{-1}$, respectively (Kannan and Lee 1996). The persistence of TPT in soils has been demonstrated with triphenyltin acetate remaining in the top layer (1 - 10 cm) of the soil column a year after application and less than 4 % of the applied TPT leaching from the soil (Loch et al., 1990).

Bioconcentration of organotins along the food chain may lead to high levels in food for human consumption. Also, a wide variety of organotins, including octyltin, butyltin and phenyltin derivatives, are in direct contact with food due to their use as stabilisers in plastic products. Organotin agrochemicals and the use of sludge amended soils could also lead to direct transfer to agricultural produce. Organotins have been detected in seafood and other foodstuffs, although concentrations detected have been reported to cause negligible risks (Kannan et al., 1995; Cardwell et al., 1999; Keithly et al., 1999; Takahashi et al., 2000; Belfroid et al., 2000). However, a world-wide effective value for an acceptable daily intake (ADI) does not exist.

1.2 Toxicity towards microorganisms

1.2.1 *General considerations*

The concentration and form of tin in the environment determines its toxicological effects. The general rule that tetraorganotins (R_4Sn) and inorganic tin are not toxic does not always apply (Cooney, 1995). Toxicity of inorganic tin to the cyanobacteria, *Synechocystis aquatilis* is dependent on pH with toxic effects occurring under extreme alkali conditions (Pawlik-Slowrońska et al., 1997). At pH 7, neither Sn(II) nor Sn(IV) exhibited any inhibiting effects at concentrations up to 10 mg Sn L^{-1}, while at pH 9.0, both the growth of the cyanobacteria and chlorophyll a content were reduced. Addition of organic moieties to inorganic tin makes the compounds more lipid soluble, thereby altering their toxic effects. The number and nature of the groups bonded to the tin atom influences toxicity. The toxicity of organotins generally decreases in the order tri- (R_3SnX) > di- (R_2SnX_2) > mono- ($RSnX_3$) substituted compounds (Hallas and Cooney, 1981a). However, this series does not hold for all microorganisms. For example, MBT was more effective than DBT at causing K^+ leakage from yeast (Cooney et al., 1989) and MBT and inorganic tin were more effective than TBT or DBT in inactivating bacteriophage T4 (Doolittle and Cooney, 1992).

Organotin toxicity correlates with total molecular surface area (TSA) (Eng et al., 1988). In this case, butyl-, propyl-, phenyl-, and pentyl-substituted compounds should be most toxic, while ethyl- and methyl-substituted organotins are expected to have less effect. There was a high correlation between TSA values of triorganotins and their toxicity towards *E. coli* and an alga, *Selenastrum capricornutum* (Eng et al., 1991). Toxicity increased in the order $Me_3SnCl < Et_3SnCl < Pr_3SnCl < Ph_3SnCl < Bu_3SnCl$. Toxicity also increases with hydrophobicity (Laughlin et al., 1985). Toxicity of organotins to the mud crab, *Rhithropanopeus harrisii* was a function of the hydrophobic characteristics conferred by the number and structure of the organic ligands.

The toxicity of organotins to microorganisms appears to be species-dependent (Table 1.3) and can also be reduced by morphological attributes such as the presence of melanised hyphae or chlamydospores (Gadd, 2000). Sensitivity of yeast to organotin and organolead compounds varied from strain to strain but TBT was the most toxic compound examined, followed by TPT and MBT (Cooney et al., 1989). Mono- and dimethyltins were the least toxic. Of nine organotin compounds tested towards the marine yeast, *Debaromyces hansenii*, only Ph_3SnCl and butylated compounds induced significant K^+ release from cells (Laurence et al., 1989). The order of toxicity of the butylated tins, as determined by K^+ release agreed with the findings of Cooney et al. (1989), the toxicity sequence being $Bu_3SnCl > BuSnCl_3 > Bu_2SnCl_2$. The overall toxicity of Ph_3SnCl was similar to Bu_3SnCl. In contrast, $MeSnCl_3$, Me_2SnCl_2, Me_3SnCl, Et_3SnBr, and Et_2SnCl_2 were shown to have little or no effect. Toxicity of organotins towards the cyanobacteria, *Plectonema boryanum*, as determined by photosynthesis inhibition, decreased in the following order: $Bu_3SnCl > Pr_3SnCl \geq Bu_2SnCl_2 \geq Ph_3SnCl > Et_3SnCl > Me_3SnCl > BuSnCl_3$ (Avery et al., 1991). A similar order of toxicity was reported for *Anabaena cylindrica*, although this organism was shown to be more sensitive (Avery et al., 1991).

Methanogens respond quite differently than aerobes. The effect of organotins on three methanogenic bacteria (*Methanococcus thermolithotrophicus, M. deltae* and *Methanosarcina barkeri*) contrasted with results for aerobic organisms (Boopathy and Daniels, 1991). IC_{50} values (concentration at which 50% inhibition occurs) were inversely proportional to the total surface area of the alkyltin molecules. Moreover, this was not due to tests being performed in reduced sulphide-containing media as the same procedures, when performed on *E. coli* under both aerobic and anaerobic conditions, yielded an inhibition pattern clearly resembling that reported by Eng et al. (1988). The toxicity of butytlin, phenyltin and inorganic tin compounds to three strains of sulfate-reducing bacteria, isolated from TBT-polluted sediment and identified as *Desulfovibrio* sp, also decreased with TSA (Lascourrèges et al,. 2000). Toxic effects on suspended anaerobic cultures were apparent at concentrations between 500 - 600 μM for $SnCl_4$, 55 - 260 μM for tri-, 30 - 90 μM for di- and 1 - 6 μM for mono-organotins.

Table 1.3 Toxicity of organotin compounds to microorganisms

Observed effect	Organism	Organotin	Reference
50% inhibition of photosynthesis by cyanobacteria	*Plectonema boryaum*	1.2 μM TBT 11 μM DBT 13 μM TPT	Avery et al. (1991)
	Anabaena cylindrica	1 μM TBT 9 μM DBT 5 μM TPT	
50% inhibition of nitrogenase activity by cyanobacteria	*A. cylindrica*	1 μM TBT 3 μM DBT 2.5 μM TPT	Avery et al. (1991)
50% inhibition of photosynthesis by marine microalgae	*Dunaliella tertiolecta* *Skeletonema costatum*	84 μM TPT 80 nM TPT	Mooney and Patching (1995)
IC_{50}[b] of yeast growth determined using density gradient plates	*Aureobasidium pullulans*	18 μM MMT 14 mM DMT 16 μM TMT 18 μM MBT 27 μM DBT 10 μM TBT 51 μM TPT	Cooney et al. (1989)
IC_{50} of organotins (i) determined by viability counts (ii) determined by thymidine uptake	Natural population from Boston Harbour sediments	(i) 98 μM MMT 64 μM DMT 33 μM TMT (ii) 38 μM MMT 205 μM DMT 152 μM TMT	Pettibone and Cooney (1988)

Observed effect	Organism	Organotin	Reference
IC_{50} of TBT to bacterial strains from sediment of Arcachon Harbour or noncontaminated environments (nc. env.)	Clostridia :		Jude et al. (1996)
	Harbour	2.1 μM TBT	
	nc. env.	2.3 μM TBT	
	Pseudomonads :		
	Harbour	70 μM TBT	
	nc. env.	70 μM TBT	
	Enterobacteria:		
	Harbour	3 mM TBT	
	nc. env.	3 mM TBT	
IC_{50} values of tin compounds for methanogenic bacteria	*Methanococcus deltae*	9 μM MPT	Boopathy and Daniels (1991)
		520 μM DPT	
		>2 mM TPT	
	Methanococcus thermolithotrophicus	5 μM MPT	
		440 μM DPT	
		>2 mM TPT	
	Methanosarcia barkei	21 μM MPT	
		500 μM DPT	
		>2 mM TPT	
GR_{25}[c] values of organotins to three sulfate-reducing bacteria	*Desulfovibrio* sp.	55-170 μM TBT	Lascourrèges et al., (2000)
		60-260 μM TPT	
		30-70 μM DBT	
		40-90 μM DPT	
		1.4-4 μM MBT	
		0.3-6 μM MPT	
		500-600 μM $SnCl_4$	

[a] MIC, minimum inhibitory concentration

[b] IC_{50}, concentration at which 50% inhibition occurs

[c] GR_{25}, concentration at which 25% reduction in mean growth rate occurs

1.2.2 *Effect of external factors on toxicity*

The availability of organotins to microorganisms is a key determinant for uptake, bioaccumulation and toxicity. Environmental variables, including temperature, pH and ionic composition can alter toxicity.

Organotin toxicity to microorganisms is altered considerably by changes in pH and NaCl concentrations. This is due to both changes in organotin solubility and speciation and alterations in microorganism physiology. Inorganic tin is toxic to the cyanobacteria, *Synechocystis aquatilis* only under extreme alkali conditions (Pawlik-Slowrońska et al., 1997). Similarly, toxicity of TBT to *Aureobasidium pullulans* and *Rhodotorula rubra* (Cooney et al., 1989) and release of K^+ from *Debaromyces hansenii* induced by butylated organotins and TPT (Laurence et al., 1989) is dependent on external pH. Organotin toxicity has generally been reported to decrease in the presence of NaCl. NaCl reduces the rate and extent of TBT-induced K^+ release from *Z. rouxii* (Cooney et al. 1989), while the toxicity of MBT, TBT and TPT to *D. hansenii* is reduced at salinity approximating to seawater levels (Laurence et al., 1989).

Media constituents can also influence organotin toxicity (Cooney and Wuertz, 1989; Cooney, 1995). Serine and hydroxyflavone enhance inorganic tin toxicity, while gelatin and humic acids increase resistance of estuarine microorganisms to inorganic tin (Hallas et al., 1982a). Complexation of tin with the smaller molecules (serine and hydroxyflavone) may facilitate transport across the membrane while larger molecules may be excluded on a size basis. When $NaNO_3$ and KNO_3 were substituted for NaCl and KCl as the medium inorganic salts, a three-fold increase in cell viability was reported (Hallas et al., 1982a). The choice of solidifying agent used in microbiological media can also influence apparent toxicity (Hallas et al., 1982a).

Different methods for measuring toxicity can lead to discrepancy between results (Jonas et al., 1984). The relative toxicity of methyltins is lower when determined by thymidine uptake as opposed to viability counts (Pettibone and Cooney, 1988). Methods that depend on the diffusion of compounds through agar cannot be used to compare the toxicity of different organotins, due to differences in solubility and therefore diffusion rates (Cooney et al., 1989).

1.3 Mechanisms of toxicity

Toxicity of organotins can occur as a result of interaction at the external cell surface or within the cell. Consequently, organotins may be regarded as membrane-active compounds with the cytoplasmic membrane as an obvious initial site of action or they may act intracellularly, following dissolution through the cytoplasmic membrane. The inhibition of microbial processes by organotins has been recorded with main effects occurring within membranes and chloroplasts or the mitochondria in eukaryotes (Gadd, 2000).

Disruption of membrane integrity may occur due to organotin binding or as a consequence of insertion into the membrane. Release of K^+ from cells, arising from increased cytoplasmic membrane permeability, has been used to monitor organotin toxicity, implicating the cytoplasmic membrane as the site of action (Cooney et al., 1989; Laurence et al., 1989; Tobin and Cooney, 1999). Effects on bacteria include dissipation of proton gradients and inhibition of ATPase, oxidation of substrates, glycolysis, solute transport and energy-linked transhydrogenase (Cooney and Wuertz, 1989).

The effects of TBT on six enzymes: ATPase, NADH oxidase, β-galactosidase, alkaline phosphatase, glucose dehydrogenase and glucose-6-phosphate dehydrogenase, on four bacteria, two Pseudomonads and two *Bacillus* sp., indicates the interactions of TBT are specific for the microorganism (Tseng and Cooney, 1995). For example, the membrane-bound ATPase activity in intact cells of *Bacillus* sp. MC24S was inhibited by TBT while the periplasmic-confined alkaline phosphatase was not affected in either *Pseudomonas* species. Glucose-6-phosphate dehydrogenase, contained in the cytosol, was not affected significantly in intact cells of *Pseudomonas* sp. BP-4, but it was inhibited in the cytosol fraction, suggesting that, in this organism, TBT does not cross the cytoplasmic membrane to reach the enzyme in the cytosol. In contrast, TBT can stimulate the cytosolic enzyme of *Bacillus* sp. MC-24S in both intact cells and the cytosol fraction. In comparison with *Pseudomonas* sp. BP-4, the action of TBT on intracellular enzymes of *Bacillus* sp. MC-24S may be related to the relative sensitivity of this organism, as the pseudomonad had a 200-fold greater EC_{50} value (concentration that causes a 50% effect).

Organotins increase the permeability of model lipid membranes. TBT increased the permeability of liposomes formed from egg phosphotidylcholine, causing efflux of dimethylarsinic (Cullen et al., 1997), while desorption of praseodymium ions from liposomes increased with organotin concentration (Gabrielska et al., 1997). Among the trialkyltins, the longer the alkyl chains of the compounds, the greater the level of Pr^{3+} release. Organotin compounds also caused membrane depolarisation of planar bilayer lipid membranes (Radecka et al., 1999). The increased length of alkyl chains of triorganotins resulted in a higher degree of membrane depolarisation, with the sequence being: methyl < ethyl < propyl < butyl < phenyl.

Organotins may become embedded at different levels in lipid bilayers, as evidenced by alterations in membrane fluidity (Ambrosini et al., 1996). TPT, DBT, and MBT affected the fluorescence anisotropy of two probes embedded at different sites in liposomes. TPT, at concentrations as low as 5 μM, had an effect only on the probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), which is localised in the hydrophobic core of the bilayer, while 40 μM TPT affected the fluorescence of 1-(4-trimethylaminophenyl-6-phenyl-1,3,5-hexatriene) (TMA-DPH) at the membrane surface. In contrast, DBT acted only at the membrane surface, while MBT was effective at each level. The hydrophobic core region has been suggested as the site of action of TBT in liposomes. Alteration in the thermotrophic characteristics of dipalmitoyl phosphotidycholine liposomes by TBT was more pronounced in the hydrophobic core region (Ambrosini et al., 1991a). Also, the action of TBT on the fluorescence polarisation of DPH and TMA-DPH in multilamellar liposomes was more marked in the core that in the head-group region (Ambrosini et al., 1991b).

Organotins also act intracellularly and processes dependent on intact internal organelles, including respiration and photosynthesis, may be disrupted (Avery et al., 1991; Mooney and Patching, 1995; Table 1.3). Triorganotins may interact with mitochondrial membranes in three ways (Blunden et al., 1984): (i) by causing gross swelling and disruption of mitochondrial membranes (ii) by acting as ionophores, facilitating hydroxyl-anion exchange and (iii) by interfering with the ATP synthase and hydrolase system.

Inhibition of nitrification by four heterotrophic bacteria, two *Bacillus* sp., an unidentified Gram positive rod and a *Pseudomonas* sp., occurred at nanomolar levels of

TBT, DBT and MBT (Miller and Cooney, 1994). In these organisms nitrification is independent of growth and is assumed to follow the pathway:

$$NH_4^+ \rightarrow NH_2OH \rightarrow\rightarrow NO_2^- \rightarrow NO_3^-$$

For each of the four organisms, TBT inhibited growth, NH_4^+ uptake, and accumulation of NH_2OH and NO_2^-. Effects on NH_4^+ uptake were deemed to be as a result of general toxicity and not due to direct inhibition of process steps. DBT inhibited NH_4^+ uptake and accumulation of NH_2OH and NO_2^- at concentrations that did not inhibit cell growth, suggesting that the effects are not merely secondary effects of general toxicity.

TPT inhibition of respiration and photosynthesis by the marine algae, *Skeletonema costatum* and *Dunaliella tertiolecta* was dependent on intracellular accumulation (Mooney and Patching, 1995). This was particularly noticeable in the less sensitive organism, *D. tertiolecta*, where inhibition of photosynthesis and respiration was often progressive over a period of 90 minutes after addition of TPT, suggesting that the slower uptake in this organism is related to greater resistance. DPT had less effect on the processes in both organisms. *D. tertiolecta* was resistant to DPT up to the limit of its solubility (0.84 mM), while DPT inhibitory levels for *S. costatum* were over one to two orders of magnitude higher than for TPT.

1.4 Biotransformation of organotins

Biotransformation of organotins largely determines their persistence and environmental fate. Both methylation and dealkylation of tin compounds can occur in nature and may result in alteration in toxicity of the compounds. No link between detoxification and resistance has been observed, although such resistance mechanisms have been reported for other organometals, most notably methylmercury (Misra, 1992). Organotins can give rise to one another by disproportionation reactions (Cooney, 1995):

$$2R_3SnX \leftrightarrow R_2SnX_2 + R_4Sn$$

Such reactions greatly complicate transformation studies and their interpretation.

1.4.1 *Methylation*

Methylation of tin compounds occurs by both abiotic and biotic mechanisms. Methylation of tin can lead to enhanced or reduced toxicity and also contributes to the biocycling of tin in the environment. Toxicity studies on microorganisms isolated from Chesapeake Bay indicated that tetramethyltin is non-toxic, while toxicity of the less methylated organotins increases with degree of methylation (Hallas and Cooney, 1981a). In general, methylation of tin increases the volatility, toxicity, lipid solubility and adsorptivity of tin and possible mobility in the environment (Cooney, 1988).

Biomethylation of tin occurs under many conditions. It has been reported in both pure and mixed cultures derived from natural sources, in nutrient medium and in a mixture of natural water plus sediment and under both anaerobic and aerobic conditions (Cooney, 1988). In most cases the reaction is slow with reaction times in the order of weeks required for completion.

$(CH_3)_3SnOH$ in anoxic estuarine sediments mixed with seawater from San Francisco Bay was converted to $(CH_3)_4Sn$ by both biologically active and autoclaved samples (Guard et al., 1981). After 80 days, 2.4% of 75 mg L^{-1} $(CH_3)_3SnOH$ was converted to $(CH_3)_4Sn$ by active sediments, 2.7 times the amount produced in autoclaved controls. A mixed inoculum of microorganisms from Chesapeake Bay sediments transformed inorganic tin ($SnCl_4.5H_2O$) to organotin compounds (Hallas et al., 1982b) and the presence of $(CH_3)_2SnH_2$ and $(CH_3)_3SnH$ was demonstrated after 14 days incubation at 25°C. The

butylmethlytins, Bu_3MeSn and Bu_2Me_2Sn were detected in aquatic sediments from Ontario (Maguire, 1984). Since tetraalkyltins are mainly used as intermediates for the synthesis of lower organic moieties and have no uses that directly lead to their dispersion in the environment, their presence is indicative of *in situ* partial degradation of butyltins, followed by methylation. A coculture of two *Bacillus* species isolated from Boston harbour sediments methylated CH_3SnCl_3 (Makkar and Cooney, 1990). $(CH_3)_3SnX$ was first detected after 21 days incubation and following 55 days incubation at 30°C, the reaction appeared to be complete, with 35% of the original 0.17 μM CH_3SnCl_3 being detected as $(CH_3)_3SnX$.

To date, the biochemical mechanisms of tin compound methylation remain unclear. Methylcobalamin (CH_3CoB_{12}) has been proposed as the methylating agent (Blunden et al., 1984), although it has been suggested that the conditions for the methylcobalamin-mediated reactions are not met in the natural environment (Cooney, 1988). Methylating agents that can function under conditions found in the environment include CH_3I, $(CH_3)_3S^+ I^-$ and $(CH_3)_3N^+CH_2COO^-$ (Guard et al., 1981).

1.4.2 *Dealkylation*

Organotin degradation involves sequential removal of organic groups from the tin atom, which does not necessarily lead to reduced toxicity towards microorganisms. Generally, abiotic degradation can occur by four mechanisms: UV irradiation, chemical cleavage, gamma irradiation and thermal cleavage (Blunden et al., 1984), though only the first two are of environmental significance (Clark et al., 1988). Photolysis in the environment occurs at a very slow rate, with reported half-lives of greater than 89 days, while chemical cleavage can occur by either nucleophilic or electrophilic attack (Clark et al., 1988). Although microbe-mediated dealkylation of organotins has been reported, little is understood about the mechanisms involved.

TBT was metabolised by microorganisms grown from an enrichment of soil and canal organisms, which were identified as the bacterium, *Pseudomonas aeruginosa* and fungal strains, *Coniophora puteana*, *Trametes versicolor* and *Chaetomium globosum* (Barug, 1981). *P. aeruginosa* degraded 40% of 2.5 mg L^{-1} TBTO, with monobutyltin derivatives as degradation products. The organism was not able to degrade MBT. While

TBT is bound strongly by sterile Toronto Harbour sediment with a desorption half-life of at least 10 months at 20°C, it can be taken up from sediment and degraded by oligochaetes (Maguire and Tkacz, 1985). A sequential debutylation pathway at 20°C in Toronto Harbour water and water-sediment mixtures with half-lives of 5 and 4 months respectively was reported.

TBT-degrading microbes may be enriched in TBT-contaminated sites. The degradation rates of TBT in harbour water from San Diego Bay were lower for clean water (<0.03 μM TBT) than those corresponding to a contaminated site (0.49 μM TBT and 0.28 μM DBT) (Seligman et al., 1986). The principal degradation product was DBT, with MBT present to a lesser extent. Complete mineralisation proceeded slowly with a half-life of 50-75 days. TBT degradation rates were slightly longer under dark conditions, suggesting that metabolism by algae may have occurred. Higher concentrations, which would be toxic to microorganisms, were not degraded in sunlight indicating that photolysis was not occurring.

Microalgae play an important role in TBT degradation in the aquatic environment. Half-lives of TBT added to estuarine samples ranged from 3-13 days with degradation rates higher for light incubations, implicating the involvement of algae (Lee et al., 1989). The half-life of TBT in nitrate-supplemented water was reduced to 1-2 days in sunlight. The dominant algae species were the diatoms *Skeletonema costatum* and *S. tropicum.* The dinoflagellate *Procentrum triestinum* also degraded TBT while, the green alga, *Dunaliella tertiolecta* and chlorophytes, *Isochrysis galbana* and *Cricosphaera ricoco* showed limited degradation capability (Lee et al., 1989). *S. costatum* converted more than 50% of 1 μg L^{-1} TBT to DBT in culture medium after 96h incubation at 4^0C (Reader and Pelletier, 1992). The half-life of TBT incubated with *Chlorella vulgaris* and *Chlorella* sp. was 60 h and 80 h, respectively (Tsang et al., 1999). *C. vulgaris* converted 27 and 41% of the original TBT to DBT and MBT, respectively, while *Chlorella* sp. resulted in 26% of TBT being converted to DBT with no MBT being detected.

A TPT–degrading bacterial strain has been isolated (Inoue et al., 2000). *Pseudomonas chloraphis* rapidly degraded 130 μM TPT, with 40 μM DPT detected after 48 h. TPT degradation was catalysed by a low molecular weight compound detected in the extract. Other fluorescent pseudomonads also showed similar TPT degradation activity,

suggesting that the extracellular compound that is produced by fluorescent pseudomonads could function as a catalyst to cometabolise TPT.

In the aquatic environment, TBT degradation is more rapid in water than in sediment (Table 1.4). This disparity may be caused by the inhibition of microbial activity at the higher TBT concentrations found in sediment layers, or even the absence of oxygen (Clark et al., 1988). In anaerobic sediments, with TBT contamination ranging from 449 to 1290 ng g^{-1}, no decrease in TBT or increase in its breakdown products was evident over a 330 day period (Dowson et al., 1993b). In contrast, TBT half-life in the aerobic sediment ranged from 360-775 days. As TBT is not completely adsorbed to particulate matter, slow release into overlying waters may occur (Stewart and de Mora, 1990). Operations such as dredging or natural disturbances such as storms may facilitate this process by transporting the sediment and resuspending it in the water column.

The mechanism(s) of organotin dealkylation by microorganisms are not known. In fish and crustaceans TBT is metabolised in two phases (Lee, 1996). The phase-one reactions involve a cytochrome P450 dependent system that hydroxylates TBT to alpha-, beta-, gamma- and delta-hydroxydibutyltin derivatives. In phase-two, these hydroxylated derivatives are conjugated to sugars and sulphate to form highly polar compounds that can be rapidly eliminated from the animal. There is some evidence to suggest the formation of hydroxybutylated intermediates by microorganisms. Hydroxylated metabolites resulted from the incubation of diatom cultures with TBT (Lee et al., 1989). Products were tentatively identified as (δ-hydroxybutyl)dibutyltin, (γ-hydroxybutyl)dibutyltin and (β-hydroxybutyl)dibutyltin.

Table 1.4 TBT degradation in the environment

Study	Initial TBT	Comments	TBT half-life
Natural waters			
Seligman et al. (1986)	0.55-2.0 μg L^{-1}	Spiked seawater from clean site, San Diego Bay	
		light incubation:	9-13 days
		dark incubation:	12-19 days
		Spiked seawater from yacht harbour	
		light incubation:	6 days
		dark incubation:	7 days
Lee et al. (1989)	0.4-1.6 μg L^{-1}	TBT-spiked estuarine seawater	3-13 days
Maguire and Tkacz (1985)		Seawater from Toronto Harbour	
	<0.1 μg L^{-1}	unspiked:	20±5 weeks
	1000 μg L^{-1}	spiked:	35±8 weeks
Seligman et al. (1988)	0.23 μg L^{-1}	Degradation of TBT in unspiked seawater	6±1.5 days
	2 μg L^{-1}	Degradation of radiolabelled TBT in spiked seawater	6-7 days
Harino et al. (1997)	9.3 μg L^{-1}	Spiked estuarine water incubated in the dark	15 days

Study	Initial TBT
Sediments	
(i) Tank experiments	
Waldock et al. (1990)	
Maguire and Tkacz (1985)	
Stang and Seligman (1986)	
Dowson et al. (1993c)	449-1290 ng g^{-1}
	449 ng g^{-1}
(ii) In situ core studies	
Sarradin et al (1995)	
Dowson et al. (1993b)	
Astruc et al. (1989)	
De Mora et al. (1989)	

Comments	TBT half-life
TBT degradation in marine sediments	28-76 weeks
TBT degradation in Toronto lake sediments	16±2 weeks
Degradation of TBT in San Diego Bay sediments	23 weeks
Degradation of TBT in surficial freshwater and estuarine marina sediments	360-775 days
Unspiked freshwater sediment	360 days
TBT profiles in sediment from a marina, Oleron Island, France	2.1 years
TBT profiles in sediment cores from East Anglia, UK.	0.91-5.21 years
TBT profiles in sediment cores from Arcachon Bay, France	360 days
TBT profiles in sediment from a marina, Auckland, NZ.	1.85 years

1.5 Organotin-resistant microorganisms

Despite the fact that organotins are used as biocides, very little is known about resistance in microorganisms. Whether the presence of tin in an environment selects for the presence of tin-resistant organisms is of importance. There was no significant correlation between total tin concentrations in sediments from Chesapeake Bay and numbers of microorganisms resistant to inorganic tin, indicating that the presence of the contaminant does not select for resistant populations (Hallas and Cooney 1981b). Similar results were reported for TBT-resistance of microorganisms isolated from TBT-polluted and nonpolluted estuarine sediments (Wuertz et al., 1991) and from contaminated coastal sediments (Jude et al., 1996). In contrast, microorganisms isolated from a TBT-polluted freshwater site were more resistant than those isolated from a nonpolluted site (Wuertz et al., 1991).

In artificially polluted seawater systems, enrichment of resistant organisms was achieved and the number of TBT-tolerant bacteria increased with exposure time (Suzuki et al., 1992). In water samples containing 40 ppm TBTCl, 90% of the viable bacteria were tolerant to TBTCl within one week. In contrast, no TBTCl-resistant organisms were present in the control sample lacking TBTCl. In water spiked with 40ppm Cd^{2+}, 50% of the population exhibited TBT-tolerance after 2 weeks, suggesting that there may be a relationship between TBT and Cd^{2+} resistance.

Plasmids play an important role in determining heavy metal resistance (Silver, 1992). The possibility of organotin resistance being plasmid-mediated was suggested when 8 of 10 estuarine and 12 of 19 freshwater isolates contained plasmid DNA (Wuertz et al., 1991). Furthermore, when plasmid pUM505, which contains genes encoding chromium resistance, was transferred from *Pseudomonas aeruginosa* to a *Beijerinckia* sp. the TBT-tolerance of the *Beijerincka* sp. increased 100-fold, from 8.4 mM to 840 mM TBT on a solid medium (Miller et al., 1995). Some TBT-resistant transconjugants lost both the plasmid and TBT-tolerance when transferred to medium lacking TBT. Among organometals, only resistance to organomercury has been previously reported to be plasmid-mediated (Misra et al., 1992).

An efflux system has been implicated in the TBT-resistance of an *Alteromonas* sp., which grew in a medium containing 125 mM TBT (Fukagawa et al., 1992). TBT-uptake by

the *Alteromonas* sp., and a TBT-sensitive organism, *Shewanella putrefaciens*, was complete after 1 h incubation. Unlike the TBT-sensitive organism, the *Alteromonas* sp. continued to thrive and the uptake of TBT decreased with growth. Protein profiles showed that 30 kDa and 12 kDa polypeptides increased dramatically when the strain was cultured in the presence of TBT. Analysis tentatively suggested that these proteins were localised in the cell membrane. The gene(s) responsible for resistance are chromosomal. A gene involved in resistance was cloned from this bacterium and one of the clones was sequenced (Suzuki and Fukagawa, 1995). The predicted protein sequence of this clone had domains in common with transglycosylases, indicating that a transgycosylase may be involved in TBT-resistance.

1.6 Uptake of organotin compounds

Organotins may act as both cationic metal ions, i.e. having a positive ionic charge, and as organic compounds in solution. Consequently, microbial uptake of organotins may occur as a result of cationic metal or lipophilic interactions. Inorganic metal uptake by microorganisms has been reviewed extensively (Hughes and Poole, 1989; Gadd, 1990; Volesky, 1990, Blackwell et al., 1995). Cell surfaces are predominantly anionic due to the presence of ionised groups such as carboxylate, hydroxyl and phosphate in the cell wall polymers. Such groups act as ligands, binding metals to the cell surface. Uptake is essentially a biphasic process. In the initial metabolism-independent step, termed biosorption, binding can be attributed to ion-exchange, adsorption, complexation, precipitation, and crystallisation within the multilaminate, microfibrillar cell wall structure (Tobin et al., 1984). Following biosorption, bioaccumulation, a metabolic-dependent transport of the metal across the membrane to the interior may occur.

As organic compounds, organotins also exhibit lipophilic interactions with cellular membranes. Uptake of lipophilic organic metal complexes by membrane diffusion mechanisms occurs in addition to or in place of the facilitated uptake of the free metal ion. The passive absorption of neutral, non-polar complexes of ionic metals is the same mechanism by which hydrophobic organic compounds are taken up (Hudson, 1998). Passive metal absorption is best known for the neutral chloride and to a lesser extent

hydroxide, complexes of Hg^{2+} and CH_3Hg^+ ions. Passive uptake of uncharged lipophilic chloride complexes is the principal accumulation route of both methylmercury and inorganic mercury in the diatom *Thalassiosira weissflogii* (Mason et al., 1996). Lipophilic, organic chelates of several divalent metal ions can also diffuse through cellular membranes. Uptake of lipophilic, organic-Cu metal complexes by *T. weissflogii* was attributed to diffusion (Phinney and Bruland, 1994). Two mechanisms were proposed to be involved: (i) diffusion of the lipophilic complex across the membrane followed by (ii) dissociation of the metal from the ligand and binding of the metal to intracellular binding sites. Hydrophobicity of metal complexes can be reported by K_{ow} values which may be used to predict membrane interactions. The K_{ow} values of $HgCl_2$ and CH_3HgCl are estimated to be 3.33 and 1.7, respectively (Mason et al., 1996), while K_{ow} values of $Cu(DDC)_2^0$ (neutral, diethyldithiocarbamate-Cu complex) and $Cu(Ox)_2^0$ (neutral, 8-hydroxyquinoline-Cu complex) were 2.8 and 2.6, respectively (Phinney and Bruland, 1994). The high K_{ow} of these compounds reflects their lipophilic character which allows diffusion though cellular membranes to occur. The K_{ow} of TBT is of similar magnitude with values from 2.3 to 4.1 between pH 4 and 7 (Arnold et al., 1997). Consequently, uptake by diffusion and lipophilic interactions should be expected tp play a significant role in TBT uptake mechanisms.

Cell wall biosorption has been suggested as the main uptake mechanism of organotins by microorganisms. Accumulation of TBT by a *Pseudomonas* sp., was not influenced by the metabolic activity of the cells and the majority of bound TBT was located in the cell envelope (Blair et al., 1982). TBT uptake by *Aureobasidium pullulans* (Gadd et al., 1990) and the cyanobacteria, *Synechocystis* PCC 6803 and *Plectonema boryanum* (Avery et al., 1993) occurred rapidly, and uptake was attributed primarily to adsorption to the cell surface with little or no intracellular accumulation. Adsorption isotherms fitted the Langmuir model (Langmuir, 1918) indicating apparent deposition of a single layer of solute molecules on the cell surface. However, TBT biosorption by the microalga, *Chlorella emersonii*, occurred in two phases (Avery et al., 1993). A rapid initial phase of cell wall adsorption was followed by a slower uptake. This was attributed to exposure of intracellular binding sites as a result of cell lysis.

Maximal uptake capacities were 564, 525, and 1050 nmol Bu_3SnCl (mg dry wt.)$^{-1}$ for *Synechocystis* PCC6803, *P. boryanum* and *C. emersonii*, respectively (Avery et al., 1993). These values are considerably lower than those reported for the fungus *A. pullulans* at 3.6 and 1.3 mmol Bu_3SnCl (mg dry wt.)$^{-1}$ for melanic and albino strains, respectively (Gadd et al., 1990). Greater biosorptive capacity of the pigmented strain was attributed to the presence of melanin.

Candida maltosa adsorbed TBT and TPT to maximum values of 0.51 and 0.65 μmol (g cells)$^{-1}$ respectively, which was accompanied by complete cell death and high levels of K^+ leakage (Tobin and Cooney, 1999). MBT and MPT were adsorbed to a lower degree, less than 0.1 mmol (g cells)$^{-1}$ in each case, with negligible changes in cell viability and K^+ release.

The extent of biosorption of triorganotins by the cyanobacteria *Synechocystis* PCC6803 and *Plectonema boryanum* and the microalga *Chlorella emersonii* increased with molecular mass, in the order triphenyltin > tributyltin > tripropyltin ≥ trimethyltin ≥ triethyltin (Avery et al., 1993). This correlates with the order of relative toxicity of the compounds to cyanobacteria, as previously reported (Avery et al., 1991). In contrast, in the butyltin series, uptake of the less toxic dibutyltin exceeded that of tributyltin in all three organisms and uptake of monobutyltin was greater than that of the more toxic tripropyltin.

Uptake of TBT is influenced by solution pH and salinity (Avery et al., 1993). For cyanobacterial species, TBT uptake was reduced at pH values greater than or lower than pH 5.5-6.5 (Avery et al., 1993). No inhibition of Bu_3SnCl biosorption was observed between 0.05 and 50 mM NaCl, but a 55-65% reduction resulted from increasing NaCl concentration from 50-500 mM. TBT uptake by *A. pullulans* was unaffected by external pH between pH 3.5 and 6.5, while an approximate 20% decrease in TBTCl biosorption resulted at pH 2.5 (Gadd et al., 1990).

1.7 Introduction to experimental work

The experimental work undertaken in this thesis serves to add to the knowledge available in the literature in a number of ways. Uptake by microorganisms has largely been overlooked when considering the fate and effect of organotins. It is important to understand such interactions because microorganisms are at the base of the food web and mediate a number of important environmental processes, including degradation of many toxic compounds. Few studies have focused on the uptake of organotins despite the fact that accumulation is a prerequisite for any subsequent toxic effects. More importantly, microbial uptake and accumulation are frequently the first step in transfer of organotins along the food chain. As organotins have been detected in both freshwater and marine ecosystems, the influence of pH and salinity on uptake and toxicity is of importance in assessing the risks of organotin pollution.

A major objective of this research was to determine the principal uptake mechanisms of inorganic tin and organotins by *Candida maltosa* and to elucidate their subsequent toxic effects. The influence of organotin speciation on these interactions is discussed in detail. The yeast *Candida maltosa*, an isolate from an asphalt refinery and its associate watershed was chosen as a model organism (Turner and Ahearn, 1970). Organotin contamination in aquatic systems occurs principally in regions with high shipping, harbours and shipyards. These are regions also associated with hydrocarbon-polluted environments, where enrichment of *C. maltosa* occurs (Mauersberger et al., 1996).

The first goal of this research was to compare the uptake of Sn(IV), TBT and TMT by *C. maltosa* at low, environmentally significant concentrations. A hydride generation atomic absorption technique was developed for the detection of the tin compounds. Toxicity of the compounds was assessed in terms of loss in cell viability and membrane damage, as indicated by K^+ release and changes in membrane fluidity. In all experiments organotin uptake and toxicity were determined after 30 minutes exposure. This time point was chosen on the basis that studies have shown that microbial organotin uptake is rapid and almost all occurs within the first 5 min of contact (Avery et al., 1993; Gadd et al., 1990). Previous published work on organotin

uptake by *C. maltosa* also used this cell exposure time (Tobin and Cooney, 1999) so selection of a 30 min period facilitated direct comparison. A single time point was chosen, as the focus of this work was to examine the influence of environmental factors on tin uptake, toxicity and resulting morphological changes. Examination of time course data was beyond the scope of this work but would merit future study.

The influence of the external environment on organotin interactions was also assessed. Aqueous speciation and lipophilicity of organotins varies with both pH and NaCl concentration. A model developed by Arnold et al. (1997) was adapted to determine organotin speciation and overall octanol-water distribution ratios (D_{ow}) between pH 3.5 and 7.5 and in 0-500 mM NaCl. The influence of pH and NaCl on TBT and TPT toxicity and the contribution of the various organotin species are discussed in detail. The following sections introduce in more detail the background to the work undertaken.

1.7.1 *Analysis of organotin compounds*

Reliable and sensitive analytical techniques are required to determine the trace environmental levels and to elucidate and understand the fate and effects of pollutants. The most sensitive methods for analysis of organotins involve the conversion to alkyl derivatives or volatile hydrides and determination with specific detectors (Fent 1996b). In the research conducted throughout this thesis, cells were exposed to organotin concentrations between 5 and 100 μM. Consequently, a sensitive, reproducible and rapid method was required for organotin analysis. Metals are routinely analysed using atomic absorption spectrometric (AAS) detection. However, the conventional AAS method of aspirating a sample into an air-acetylene flame has limited effectiveness for organotin analysis due to the inherent insensitivity of the technique. A significant increase in analytical sensitivity is obtained when tin compounds are analysed by flame AAS following conversion to volatile hydrides, a technique known as hydride generation AAS (HGAAS). A sensitive HGAAS method was developed for analysis of Sn(IV), TBT and TMT. Conversion of TPT to the hydride form is poor and therefore HGAAS analysis is not suitable. Instead, TPT was analysed using a polarographic method.

The use of HGAAS for the determination of several hydride-forming elements is well established. It offers high sensitivity, low cost, reliability, high speed, convenience and simplicity. The technique allows the selective separation of the analyte from the matrix enabling most interfering species to be avoided and pre-concentration of the analyte to occur. This results in greater sensitivity and suppression of interference during atomisation.

The hydride technique can be divided into three steps: hydride generation and release, hydride transport and hydride atomisation.

Hydride generation and release can be defined as the conversion of an analyte in the acidified sample to the corresponding hydride and its transfer to the gaseous phase. The reaction can be classified as either metal/acid reduction or sodium tetrahydroborate ($NaBH_4$)/acid reduction. The latter reduction method is the most popular and the reaction occurs as follows:

$$NaBH_4 + 3H_2O + HCl \Rightarrow H_3BO_3 + NaCl + 8H + E^{m+} \Rightarrow EH_n + H_2$$

where, E is the analyte element and m may or may not equal n.

For acidification, HCl is generally used. Optimum conditions for hydride generation depend on type of analyte and the valency of the element. Parameters such as volume and concentration of acid and reducing agent, type of atomisation cell and its temperature all affect the signal obtained.

There are two modes of hydride transfer: direct transfer and collection. In direct transfer, including continuous flow, flow injection and batch, the hydride release from the sample solution is directly transferred to the atomiser. In collection mode, the hydride is collected in a collection device until the evolution is complete and then it is transferred to the atomiser. In flow injection hydride generation AAS, acid and reducing solution flow continuously at a constant rate to the gas/liquid separator and a limited volume of sample is injected into the acid stream. The signal produced is transient. The flow injection method offers the advantages of being very simple, rapid and precise.

The atomisation of hydride takes place in the optical beam of the AAS. Externally heated quartz tubes are by far the most common mode of atomisation as the technique is simple and sensitive.

For this study, flow injection HGAAS was chosen as the desired method of organotin analysis. A conventional AAS system was adapted as shown in Figure 1.2. Reduction of tin occurs at the T-piece and the reaction is completed by the time the flow reaches the gas/liquid separator. At this point, the liquid products flow via a U-tube to a free running drain, while the gaseous products are purged by argon into the atomisation cell.

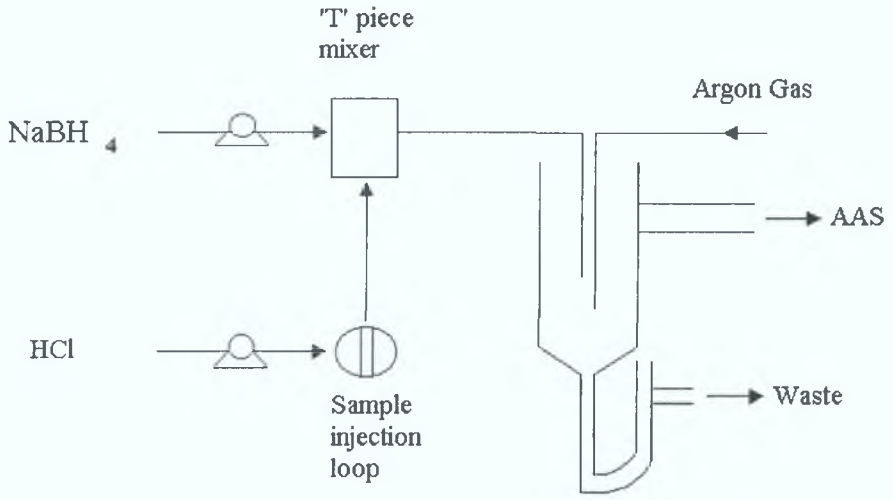

Figure 1.2 Schematic of a flow injection hydride generation AAS system

1.7.2 *Fluorescence anisotropy as an indicator of membrane fluidity*

Release of K^+ from cells, arising from increased cytoplasmic membrane permeability, has been used to monitor organotin toxicity, implicating the cytoplasmic membrane as a site of action (Laurence et al., 1989; Tobin and Cooney, 1999). Alterations in membrane fluidity, as indicated by changes in anisotropy of fluorescent probes embedded in membranes, may also be used as an indicator of membrane interactions and gives an indication of the localisation of compounds within membranes.

1.7.2.1 Theory of fluorescence anisotropy and membrane fluidity

Membrane fluidity can be described as the various motions, as well as the degree of packing of the membrane components. Methods available for determining membrane fluidity include fluorescence polarisation, Raman spectroscopy, electron spin resonance

and nuclear magnetic resonance. In this study, the anisotropy of the fluorescent probes, DPH and TMA-DPH were used to monitor membrane fluidity. The fluorescence polarisation technique has several advantages. It can be readily applied to complex systems including cellular membranes, the polarised signal is highly sensitive and reproducible and data are promptly interpretable (Shinitsky and Barenholz, 1978). The method involves labelling membranes with the fluorophores and then exciting the molecules with polarised monochromatic light. According to the movement of the probe, which is dependent on the fluidity of the immediate surroundings, the emitted light will be partially depolarised with respect to the plane of polarisation of the excitation light (Figure 1.3).

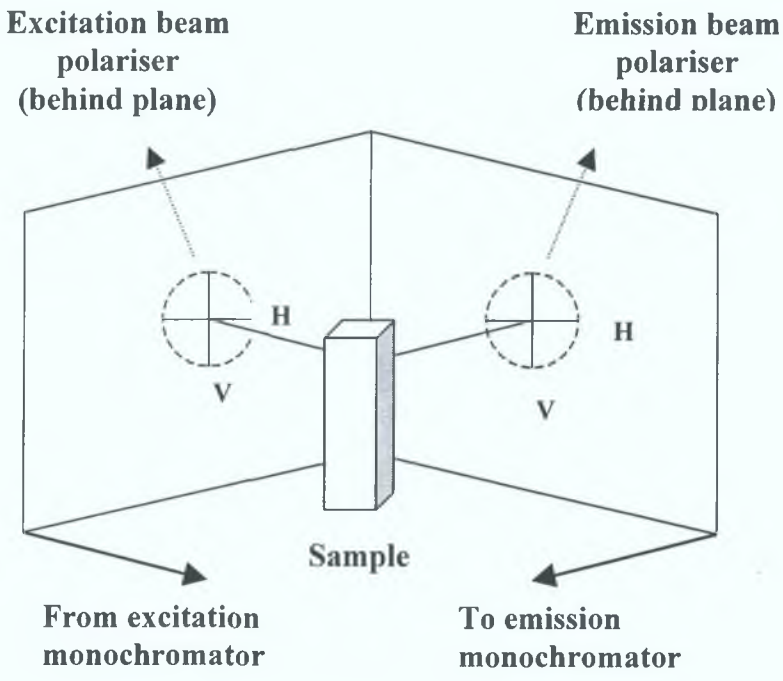

Figure 1.3 Schematic of an optical system for measurement of fluorescence anisotropy

In order to measure fluorescence anisotropy, a sample is excited with vertically polarised light. The emission intensity is measured parallel, I_{VV} and perpendicular, I_{VH} to the vertical plane of the polarisation of the excitation light.

When a non-polarising sample is excited with horizontally polarised light, the emission components, I_{HV} and I_{HH} should be equal and hence,

$$\frac{I_{H_V}}{I_{H_H}} = 1$$

where,

I_{HV} = emission intensity of vertically polarised light perpendicular to the plane of excitation and

I_{HH} = emission intensity of horizontally polarised light parallel to the plane of excitation.

However, the effect of the instrument response is to cause the ratio to deviate from unity to a value, G, the instrument correctional factor. This is measured by exciting the sample with horizontally polarised light and measuring the parallel and perpendicular components. G is given by the equation

$$G = \frac{I_{H_V}}{I_{H_H}}$$

Fluorescence anisotropy can then be calculated by the following equation

$$A = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + 2GI_{V_H}}$$

where,

I_{Vv} = emission intensity of vertically polarised light parallel to the plane of excitation and

I_{VH} = emission intensity of horizontally polarised light perpendicular to the plane of excitation.

For vertical excitation light, I_{Vv} will decrease and I_{VH} increase if rotation of the probe occurs. Hence, the greater the extent of rotation of the fluorophore during the lifetime of its electronic state, the smaller will be the observed fluorescence intensity. Consequently, an inverse relationship exists between membrane fluidity and anisotropy.

1.7.2.2 Fluorescent probes – DPH and TMA-DPH

The use of DPH and its charged derivative, TMA-DPH, as fluorescent probes for monitoring membrane fluidity is widely documented (Kuhry et al., 1983 and 1985; Shinitsky and Barenholz, 1978). DPH is one of the most efficient fluidity probes

available for the hydrocarbon region of lipids and the fluorescence anisotropy reflects, almost exclusively, the angular displacement of the molecular axis (Shinitsky and Barenholz, 1978). DPH can be dispersed in aqueous media to form microaggregates, which are practically void of fluorescence. When such a dispersion is mixed with a lipid-containing system, the probe incorporates into the hydrophobic domain which results in a sharp increase in the fluorescence signal. DPH is generally used with isolated membranes but may be used with protoplasts (Alexandre et al., 1994) and intact cells (Baut et al., 1994; Swan and Watson, 1997). In cells, DPH is distributed between the hydrophobic region of lipidic membranes (Kuhry et al., 1983) and DPH anisotropy reflects the average fluidity of all cellular membrane lipids (Swan and Watson, 1997).

The cationic derivative of DPH, TMA-DPH is incorporated rapidly into the cytoplasmic membrane of cells and remains restrictively localised in the hydrophilic head region of lipids (Kuhry et al., 1983). The length of the hydrophobic part of TMA-DPH is less than that of a 10-carbon aliphatic chain, so it is less sensitive to modification at the hydrophobic core membrane bilayers.

TBT, TPT, DBT and MBT affected the fluorescence anisotropy of DPH and TMA-DPH embedded at different sites in liposomes (Ambrosini et al., 1991a; Ambrosini et al., 1996). Similar fluorescence probe studies have demonstrated that inorganic metals may influence plasma membrane fluidity (Fodor et al., 1995; Aβmann et al., 1996). Cd^{2+} caused a prolonged fluidisation of energised *Schizosaccharomyces pombe* cells (Aβmann et al., 1996). In contrast, membrane fluidity of deenergised cells remained stable, indicating that the inorganic metal exerted its effect only after metabolic-dependent accumulation. Investigation of the interactions of organotins and intact cells, as opposed to liposomes, is undertaken to determine if organotins can interact with the cytoplasmic membrane solely as a consequence of their lipophilic nature. The use of two probes located at different levels in the cytoplasmic membrane will elucidate the localisation of organotins within membranes.

1.7.3 *Uptake and toxicity of inorganic tin and organotins*

Knowledge of the effects of tin compounds at low, environmentally relevant concentrations is generally lacking, as previous reports have mainly focused on

concentrations in the range 0.08-5 mM (Gadd et al., 1990; Avery et al., 1993; Tobin and Cooney, 1999). Since TBT legislation was introduced, maximum concentrations rarely exceed 100 ng L^{-1} along the English Channel and Atlantic coasts and 200 ng L^{-1} along the Mediterranean coast (Alzieu, 1998). However, TBT persists at high concentrations in sediments where degradation rates are often in the order of years. In harbour sediments average concentrations generally range between 1 and 2 mg (kg dry wt.)$^{-1}$ (Alzieu, 1998). Mechanical operations such as dredging and natural disturbances such as storms and bioturbation may facilitate release of TBT into the overlying water.

One of the purposes of this work was to compare the toxicity and sites of action of inorganic tin, Sn(IV) and two organotins with very different total molecular surface area at concentration ranges approximating those found in polluted waters. Also, the importance of cell wall binding versus lipophilic diffusion of organotin compounds was examined by comparison of intact cells and protoplasts. Initial tin concentrations ranged from 5 μM to 100 μM. For TBT, these concentrations ranged from non-toxic to levels that result in complete loss in cell viability, allowing the fate and effect in viable cells to be determined.

1.7.4 *Influence of environmental conditions on organotin interactions*

The assessment of metal pollution requires an understanding of the various processes that influence the bioavailability and toxicity of the contaminant. Solution pH, ionic composition and strength and temperature influence the chemical speciation of organotins in the aquatic environment. TBT and TPT solubility also varies with pH, with a minimum solubility in the range pH 6-8 and decreases with increasing salt concentration (Table 1.5).

Table 1.5 Aqueous solubility of TBT and TPT at 25°C (adapted from Inaba et al., 1995)

Compound	Solvent	Solubility (μM)		
		pH 5[a]	Minimum[b]	pH_{min}[c]
TBTCl	Distilled water	625	134	7.9
	Seawater	17.9	8.9	7.3
TPTCl	Distilled Water	35.7	7.1	7.2
	Seawater	8.9	2.7	7

[a] Solubility at pH 5.0

[b] Minimum solubility value

[c] pH at which minimum solubility was determined

One of the main aims of this research was to examine the influence of solution pH and NaCl concentration on the uptake and toxicity of the triorganotins, TBT and TPT. Organotin compounds undergo pH-dependent hydrolysis when introduced into water:

$$R_3Sn^+ \leftrightarrow R_3SnOH + H^+$$

where the hydrolysis constant, K_a is given by

$$K_a = \frac{[R_3SnOH][H^+]}{[R_3Sn^+]}$$

Thus, the formation of cationic and neutral species in solution varies with pH, with the fraction of cationic species dominating below the pK_a and fraction of neutral hydroxide species above the pK_a. The pK_a values of TBTOH and TPTOH have been reported as 6.25 and 5.20 respectively, while the pK_a of TMT is in the range 5.79 to 6.60 (Arnold et al., 1997).

In seawater, TBT is composed of tributyltin chloride (TBTCl), tributyltin hydroxide (TBTOH), and an aquo complex ($TBTOH_2^+$) (Laughlin et al., 1986). The equilibrium distribution is influenced by Cl^- concentration, dissolved CO_2 and pH. The presence of a tributyltin carbonato ($TBTOHCO_2^-$) species has been suggested (Laughlin et al., 1986), but this is questionable due to the low concentration of HCO_3^- in seawater

and is assumed to be an impurity in the original solution (Arnold et al., 1997). A model has been described for prediction of the aqueous speciation and D_{ow} of TBT and TPT under different ionic and pH conditions (Arnold et al., 1997). For the work described here, this model was used to predict organotin speciation and D_{ow} between pH 3.5 and 7.5 and in concentrations of up to 500 mM NaCl at pH 5.5.

Generally, it has been reported that interactions between TBT and microorganisms are reduced in the presence of NaCl and at low and high pH (Laurence et al., 1989; Cooney et al., 1989; Gadd et al., 1990; Avery et al., 1993). NaCl may alter organotin toxicity in three ways: (i) Na^+ can reduce interaction of the organotin with the cell surface by competing for binding sites or interacting with the compound itself (Cooney et al., 1989), (ii) The membrane-lipid composition may be altered, making the cells more resistant to membrane-acting compounds (Cooney et al., 1989) and (iii) Cl^- can inhibit the solubility of tributyltin compounds by association with the cation to form covalent organotin chloride (Blunden et al., 1984). In seawater (~ 0.5 M NaCl) the solubility of TBT and TPT is less than 20 μM (Inaba et al., 1995). Despite this, most of the reports to date have examined concentrations greater than 50 μM which exceed the limit of solubility (Cooney et al., 1989; Laurence et al., 1989; Avery et al., 1993). Consequently, the reduction in TBT toxicity and uptake which has been attributed to the presence of NaCl, may be due to the formation of insoluble tributyltin chloride which would reduce the availability of TBT in solution.

In the present work, the variation in TBT and TPT uptake and toxicity between pH 3.5 and 7.5 and in concentrations of up to 500 mM NaCl was investigated. These compounds were chosen for two reasons. Firstly, they are among the most toxic of organotin compounds and are routinely detected in the environment. Secondly, they are structurally very different (Figure 1.4) but have similar lipophilic properties. The total molecular surface area (TSA) of TBT is 348 $Å^2$ compared to 330 $Å^2$ for TPT while the log K_{ow} values of the hydroxide species are 4.10 and 3.53 for TBTOH and TPTOH, respectively (Arnold et al., 1997). The toxicity of the individual TBT and TPT species and the influence of lipophilicity, as determined by the model of Arnold et al., (1997) on interactions are discussed in detail. The implications of these findings on the understanding and prediction of organometal toxicity are also assessed.

TMT, TSA=150 Å^2 **TBT**, TSA=348 Å^2 **TPT**, TSA=330 Å^2

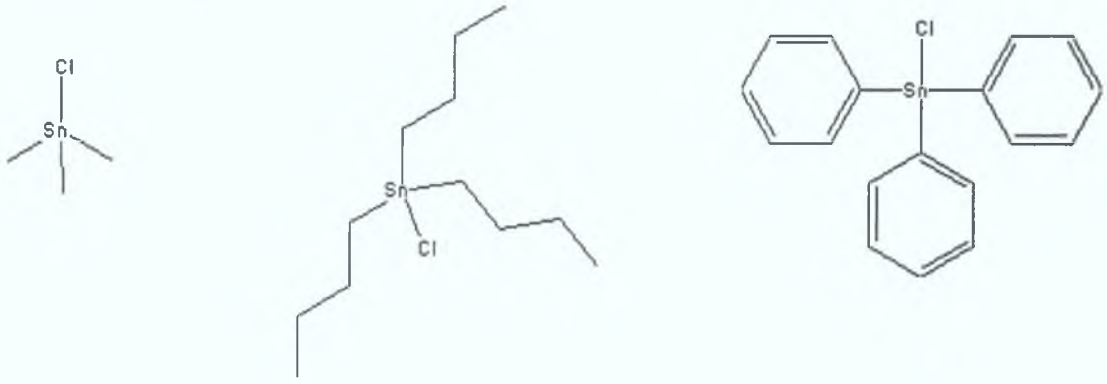

Figure 1.4 Chemical structure of TMT, TBT and TPT. Total molecular surface area (TSA) values were obtained from Eng et al (1991).

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1 Organism, culture conditions, reagents and metal analysis

2.1.1 *Candida maltosa culture conditions*

Candida maltosa R42 was supplied by S.A. Crow of Georgia State University (Atlanta, GA) and was routinely maintained on a yeast extract-peptone medium (YEPD), pH 5.5, which contained (g L^{-1}): yeast extract, 5; bacteriological peptone (Oxoid, Hampshire, UK), 5; glucose, 10; and No. 1 bacteriological agar (Oxoid, Hampshire, UK), 17.

For experimental purposes, *C. maltosa* was grown in 100 ml YEPD (less agar) in 250 ml Erlenmyer flasks at 30°C on a rotary shaker at 150 rpm. Cells were harvested in the exponential phase by centrifugation (300 x g, 5 min) and washed three times with deionised water. Cells were used within three hours of harvesting.

2.1.2 *Reagents*

All chemicals were obtained from Sigma-Aldrich (Dorset, UK) unless stated otherwise. $SnCl_4.5H_2O$, tributyltin chloride [$(C_4H_9)_3SnCl$] and triphenyltin chloride [$(C_6H_5)_3SnCl$] stock solutions were prepared in methanol (HPLC grade, Labscan Ltd., Ireland) , while trimethyltin chloride [$(CH_3)_3SnCl$] was prepared in deionised water. Fresh 1% (w/v) sodium tetraborohydrate solution was prepared daily by dissolving $NaBH_4$ in 1% (w/v) NaOH and filtering through a 0.45 μm membrane filter (Cellulose nitrate membrane filters, Whatman, Maidstone, UK). Fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylaminophenyl-6-phenyl-1,3,5-hexatriene) (TMA-DPH) were prepared as $2x10^{-4}$ M stock solutions in N,N-dimethylformamide, stored at 4°C and used within one month. KCl and NaCl salts were reagent grade from Riedel-de-Haën (Seelze, Germany). 2-(*N*-morpholino)ethanesulfonic acid (MES), piperazine-*N,N'*-bis(2-ethansulfonic acid) (PIPES) and Tris(hydroxymethyl)amino-methane (Tris) were used for buffer solutions.

2.1.3 *Metal analysis*

2.1.3.1 K^+ analysis (AAS)

K^+ was analysed using a Perkin-Elmer 3100 atomic absorption spectrophotometer (Perkin Elmer, Nerwalk, Ct., USA), fitted with a 10 cm single slot burner head, with an air-acetylene flame. K^+ concentrations were determined, after dilution to a suitable concentration, by reference to appropriate standard solutions.

2.1.3.2 Sn(IV), TBT and TMT analysis (HGAAS)

TBT, Sn(IV) and TMT concentrations were determined using a flow injection hydride generation system in combination with a Perkin-Elmer 3100 AAS. The hydride system was based on that described by Dunne (1994) and consisted of the following (Fig. 1.2): two peristaltic pumps, a 3-way connector T-piece mixer (Omnifit, Cambridge, UK), a gas/liquid separator (Plasma-Therm, London, UK) and a manual sample injection valve with 0.5 ml sample loop, consisting of Teflon tubing of 1 mm i.d.(Omnifit). This tubing was also used throughout the flow injection system. The tube lengths from the sample injection loop to the T-piece mixer and from the T-piece to the separator were 27 and 16 cm, respectively. 1% (w/v) Sodium tetraborohydrate and 1 M HCl were pumped at 4.0 and 4.8 ml min^{-1}, respectively. The AAS burner head was modified to allow a silica atomisation cell (T-shaped silica tube, 150x2 mm i.d.) to be supported in an air-acetylene flame approximately 5 mm above the slot of a 10 cm single-slot burner head. Argon (Air Products, Ireland) was used as the carrier gas to purge the hydrides from the separator into the T-tube. Samples were diluted with an equal volume of 2 M HCl prior to injection and tin concentrations were determined by reference to corresponding standard solutions. A minimum of 1 ml sample diluted with 1 ml 2M HCl was required for analysis.

In the hydride generation system, reduction of tin by sodium tetrahydroborate occurs at the T-piece and the reaction is completed by the time the flow reaches the gas/liquid separator. At this point, the liquid products flow via a U-tube to a free-running drain, while the gaseous products are purged by argon into the atomisation cell. In the operation of the system, two sampling cycles were used. In the first, the acid and

$NaBH_4$ streams were allowed to mix at the T-piece and the peak height signal set to zero. During this period, the hydrogen generated enabled a blank level to be monitored. The second period involved the injection of the sample via a four-way rotary valve into the acid carrier stream. The peak height signal was measured and recorded. At the end of this period, the rotary valve was switched back to the injection position and the cycle was repeated. This sequence of events does not include a specific time period for washing the system, as experimentation had shown that, in the period immediately after the analysis, the signal had returned to the baseline. This ensured that the blank level was achieved between each cycle and that within each cycle, the analyte was measured above the blank level.

2.1.3.3 TPT analysis (differential pulse polarography)

TPT concentrations were determined using a model 394 electrochemical trace analyser with a 303A static mercury drop electrode (EG&G Instruments, Princeton Applied Research). Differential pulse polarography (pulse amplitude 50 mV) and a hanging mercury drop working electrode (HMDE) were employed for analyses and the peak for TPT was obtained at a half-wave potential of approximately -0.69 V.

Samples (maximum of 5 ml) were diluted to 50 ml in support electrolyte consisting of 0.16 M NH_4Cl in 40% (v/v_{aq}) ethanol and adjusted to pH 2.5 using 2M HCL. For analysis, samples were deaerated by purging with N_2 for 3 min and each sample was analysed three times with a 30 s N_2 purge between each reading. TPT concentrations were determined by reference to appropriate standard solutions.

2.1.3.4 TBT and TPT solubility

The effect of NaCl and pH on the solubility of TBT and TPT was determined using the method of Inaba et al (1995). Excess amounts of organotin was added to flasks containing test solutions and equilibrated by shaking at 150 rpm. After dissolution equilibrium was established samples were centrifuged for 5 min at 2500 x g. Samples were diluted and the concentration of soluble organotin analysed. Solubility at pH 5.5 in 0 and 500 mM NaCl and at 7.5 was determined.

2.2 Uptake and toxicity of Sn(IV), TBT and TMT at pH 5.5

Uptake of Sn(IV), TBT and TMT by intact cells and protoplasts of C. maltosa at pH 5.5 was compared. Subsequent loss in cell viability was assessed. Interactions of the organotins with cellular membranes was also investigated by monitoring K^+ release and membrane fluidity.

2.2.1 *Preparation of protoplasts*

Yeast cells (~0.5 g wet weight) were suspended in 4 ml of 10 mM tris-HCl, 10 mM dithiothreitol, 5 mM EDTA, pH 8 buffer. The suspension was incubated at 37°C for 30 min on an orbital shaker at 150 rpm. Cells were centrifuged (2500 x g, 5 min) and washed three times with 5mM PIPES, 1.2 M sorbitol, pH 6.5 buffer. Cells were incubated in the same buffer with 10 mg ml^{-1} Novozym-234 (Calbiochem-Novobiochem, Nottingham, UK) and incubated at 25°C on an orbital shaker at 120 rpm. Protoplast formation was monitored by comparing the optical density at 610 nm of samples diluted with deionised water and was complete within 3 h. Protoplasts were collected by centrifugation (2500 x g, 5 min) and suspended in 10 mM MES, 1.2 M sorbitol, pH 5.5 buffer. Conversion of whole cells to protoplasts was confirmed to be ≥ 99% by counting with a haemocytometer the number of intact cells remaining after dilution in deionised water and comparing this to the number of cells after dilution with sorbitol-MES buffer.

2.2.2 *Organotin uptake experiments*

Both protoplasts and intact cells were washed three times with 10 mM MES, pH 5.5 buffer (containing 1.2 M sorbitol in the case of protoplasts) and suspended in 50 ml buffer to an approximate cell density of $1x10^7$ cells ml^{-1}. After 30 min equilibration by shaking at 150 rpm at room temperature, organotin (from a stock solution) was added to the desired concentration. Tin-free methanol controls were also investigated, as TBT and $SnCl_4$ stock solutions were prepared in methanol. 2% (v/v_{aq}) methanol, corresponding to the highest concentration present in tin solutions, was examined. After 30 min incubation, 5 ml samples were removed and the biomass separated by centrifugation (2500 x g, 5 min). Supernatants were retained for K^+ and tin analysis. In the case of

protoplasts, cell pellets were retained for viability determination using methylene blue staining. For determination of intact cell viability, 1 ml cell suspension samples were removed from flasks.

2.2.3 *Cell viability measurements*

2.2.3.1 Viability of intact cells

In all experiments, viability of intact cells was monitored by growth on YEPD plates. Suspension samples were serial diluted in sterile 1% bacteriological peptone, pH 5.5, and plated in duplicate on YEPD agar. Colonies were counted after incubation at 30°C for 48 h. Viability was defined as the ratio of experimental to control counts, expressed as a percentage.

2.2.3.2 Viability of protoplasts

Protoplast viability was determined using a modified methylene blue staining technique. The staining solution was prepared in the following manner: 0.01 g of methylene blue was dissolved in 10 ml deionised water, to which 1 g of tri-sodium citrate and 21.87 g of sorbitol was added. A further 60-70 ml of water was added, the solution mixed and filtered through Whatman No.1 filter paper and made up to 100 ml with deionised water.

Protoplast pellets were washed twice with MES-sorbitol buffer and resuspended to a suitable cell density. 0.5 ml of protoplast suspension was mixed with 0.5 ml staining solution and incubated for 5 min prior to counting with an improved Neubauer haemocytometer. Dead cells stained blue while viable cells remained clear. For all experiments, viability of original protoplast suspensions exceeded 99%, so cell viability was expressed as the percentage of clear cells to total number of cells counted. A minimum of 250 cells was counted in all cases.

2.2.4 *Membrane fluidity measurements*

Membrane fluidity was monitored by measuring the steady-state fluorescence anisotropy of TMA-DPH or DPH incorporated into intact cells or protoplasts, using a Perkin Elmer

LS 50 Luminescence Spectrometer (Buckinghamshire, UK) fitted with horizontal and vertical polarisers.

Intact yeast cells and protoplasts were suspended in 50 ml of 10 mM MES, pH 5.5 buffer (containing 1.2 M sorbitol in the case of protoplasts) to an approximate cell density of $1x10^7$ cells ml^{-1}. TMA-DPH or DPH, from 0.2 mM stock solutions in dimethylformamide, was added to cell suspensions to a final concentration of 0.5 μM. After 30 min equilibration by shaking at 150 rpm, tin from a stock solution was added to the desired concentration. Controls with 2% methanol, less metal were also examined. Fluorescence intensity and anisotropy readings were recorded after 30 min incubation. The excitation and emission wavelengths for both probes were 360 and 450 nm, respectively.

To ensure that steady state fluorescence was measured during the time range of the experiments, partitioning of the probe molecules in cells was monitored 60 min. Cells, prepared as outlined in section 2.1, were suspended in 50 ml 10 mM MES, pH 5.5 to a cell density of $1x10^7$ cells ml^{-1}. Probe was added and fluorescence intensity and anisotropy monitored at regular intervals. The contribution of light scattering to fluorescence measurements was determined as described by Kuhry et al. (1985). Briefly, the scattered light intensity and anisotropy were determined by measuring an unlabelled control under the same conditions as the sample. The contribution of light scattering was calculated as outlined in Section 3.2.

2.3 Influence of pH on TMT uptake and toxicity

C. maltosa was grown in 100 ml YEPD in 250 ml Erlenmyer flasks at 30°C on a rotary shaker (150 rpm). Cells were harvested in the exponential phase by centrifugation (300 x g, 5 min) and washed twice with deionised water followed by three washings with 10 mM MES (pH 3.5, 4.5 and 5.5) or 5 mM PIPES (pH 7.5) buffers. Yeast cells were suspended in 50 ml of the corresponding MES or PIPES buffers to an approximate cell density of $1x10^7$ cells ml^{-1}. After 30 min equilibration by shaking at 150 rpm at room temperature, TMT (from a stock solution) was added to the desired concentration. After 30 min incubation, 5 ml samples were removed and the biomass separated by

centrifugation (2500 x g, 5 min). Supernatants were retained for K^+ and tin analysis. Cell suspension samples were also obtained for viability studies.

2.4 Influence of pH and NaCl concentration on TBT and TPT uptake and toxicity

Accumulation of TBT and TPT by C. maltosa and toxic effects under various aqueous conditions was examined. The influence of pH (between pH 3.5 and 7.5) and the effect of NaCl, up to 500 mM, at pH 5.5 were investigated. Initial organotin concentrations in test solutions ranged up to the limit of solubility as determined under the specified conditions.

2.4.1 *Preparation of biomass*

C. maltosa was grown in 100 ml YEPD as described previously. Cells were harvested in the exponential phase by centrifugation (300 x g, 5 min) and washed twice with deionised water followed by three washings with 10 mM MES (pH 3.5, 4.5 and 5.5) or 5 mM PIPES (pH 7.5) buffers. Where specified, NaCl was added to the desired concentration prior to washing cells.

2.4.2 *Effect of NaCl on C. maltosa*

The effect of NaCl itself on *C. maltosa* was determined. After harvesting, cells were washed three times with 10 mM MES, pH 5.5 buffer and suspended in 50 ml buffer to a cell density of $1x10^7$ cells ml^{-1}. After 30 min shaking on an orbital shaker at 150 rpm at room temperature, NaCl was added to give the desired concentration. After 30 min incubation, 5 ml samples were removed and the biomass separated by centrifugation (2500 x g, 5 min). Supernatants were retained for K^+ and Na^+ analysis. Cell suspension samples were also obtained for viability studies.

The influence of NaCl on fluorescence anisotropy of DPH and TMA-DPH was also examined. Cells were suspended in 50 ml 10 mM MES, pH 5.5 buffer to a concentration of $1x10^7$ cells ml^{-1}. DPH or TMA-DPH was added to a final concentration of 0.5 μM. After 30 min, NaCl was added to give a concentration of 50 or 500 mM NaCl and incubated for a further 30 min. Fluorescent anisotropy was determined as described in section 2.2.4.

2.4.3 *Exposure to organotins*

Yeast cells were suspended in 50 ml 10 mM MES or 5 mM PIPES buffers (with NaCl added where specified) to an approximate cell density of $1x10^7$ cells ml^{-1}. After 30 min equilibration by shaking at 150 rpm at room temperature, TBT or TPT (from stock solutions) was added to the desired concentration. After 30 min incubation, 5 ml samples were removed and the biomass separated by centrifugation (2500 x g, 5 min). Supernatants were retained for K^+ and tin analysis. Cell suspension samples were also obtained for viability studies.

2.4.4 *Membrane fluidity measurements*

Cells were harvested in the exponential phase by centrifugation (300 x g, 5 min) and washed twice with deionised water followed by three washings with 10 mM MES (pH 3.5, 4.5 and 5.5) or 5 mM PIPES (pH 7.5 and 8.0) buffers. Where specified, NaCl was added to the desired concentration prior to washing cells. Yeast cells were suspended in either 50 ml 10 MES or PIPES buffers to an approximate cell density of $1x10^7$ cells ml^{-1}. The influence of NaCl and pH on the effects of TBT on DPH and TMA-DPH anisotropy was assessed using the procedure outlined previously (Section 2.2.4).

2.5 Theoretical considerations: Aqueous speciation and 1-octanol-water partitioning of TBT and TPT

Using the following model (adapted from Arnold et al., 1997) the aqueous speciation and 1-octanol-water distribution ratio of TBT and TPT could be calculated as a function of pH and anion concentration.

2.5.1 *Aqueous Speciation*

The fractions $\alpha_{R_3Sn^+}$, α_{R_3SnOH}, and $\alpha_{R_3SnX_i}$ of different triorganotin (TOT) species present at a given pH and solution composition may be expressed as:

$$\alpha_{R_3Sn^+} = \frac{1}{1+10^{pH-pK'_a}+\sum_i K'_i[X_i^-]} \qquad [1]$$

$$\alpha_{R_3SnOH} = \frac{10^{pH-pK'_a}}{1+10^{pH-pK'_a}+\sum_i K'_i[X_i^-]} \qquad [2]$$

$$\alpha_{R_3SnX_i} = \frac{K'_i[X_i^-]}{1+10^{pH-pK'_a}+\sum_i K'_i[X_i^-]} \qquad [3]$$

Here, K'_a is the mixed acidity constant of the dissociation reaction:

$$R_3Sn^+(aq) \leftrightarrow R_3SnOH + H^+ \qquad [4]$$

and is given by

$$K'_a = \frac{[R_3SnOH]\{H^+\}}{[R_3Sn^+]} = K_a \frac{f^c_{R_3Sn^+}}{f^n_{R_3SnOH}} \qquad [5]$$

where K_a is the mixed acidity constant at infinite dilution, $\{H^+\}$ is the activity of the proton and $f^c_{R_3Sn^+}$ and $f^n_{R_3SnOH}$ are the activity coefficients of R_3Sn^+ and R_3SnOH, respectively, for the given solutions conditions.

The K'_i values represent the formation constants of R_3SnX_i due to exchange of a water molecule by X_i^-, a monovalent anion (excluding OH^-):

$$R_3Sn^+(aq) + X_i^- \leftrightarrow R_3SnX_i + H_2O \qquad [6]$$

$K_i^{'}$ is defined as

$$K_i^{'} = \frac{[R_3SnX_i]}{[R_3Sn^+][X_i^-]} = K_i \frac{f^c_{R_3Sn^+} f^c_{X_i^-}}{f^n_{R_3SnX_i}} \qquad [7]$$

where, K_i is the corresponding equilibrium constant at infinite dilution and the f_i values are the activity coefficients of the species under the given solution conditions.

For any charged species *i* (i.e. R_3Sn^+, X_i^-) the activity coefficient in aqueous solution can be estimated using the Davies equation:

$$\log f_i^c = \frac{-z_i^2}{2}\left[\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3I\right] \qquad [8]$$

where, f_i^c is the activity coefficient of the charged species *i*, z_i is its charge and *I* is the ionic strength.

For uncharged TOT species (i.e. R_3SnOH, R_3SnX_i) the effect of a given salt on the activity coefficient in water f_i^n, can be determined by

$$f_i^n = 10^{K^S [salt]} \qquad [9]$$

where, K^S is the Setschenov or salting constant for a given electrolyte and [salt] is the total molar concentration of the electrolyte.

2.5.2 *D_{ow} (Overall octanol-water distribution ratio) of TOT compounds*

The partitioning of TOT can be described by the overall distribution ratio (D_{ow}), that is defined as the quotient of the sum of the concentrations of the different charged and neutral species in 1-octanol, $[TOT]_{total, oct}$ and water, $[TOT]_{total, aq}$, respectively.

D_{ow} values of a given TOT may be expressed by

$$D_{ow} = \frac{[TOT]_{total, oct}}{[TOT]_{total, aq}} = \alpha_{R_3SnOH} K^{'}_{ow(R_3SnOH)} + \sum_i \alpha_{R_3SnX_i} K^{'}_{ow(R_3SnX_i)} \qquad [10]$$

where, $K'_{ow(R_3SnOH)}$ and $K'_{ow(R_3SnX_i)}$ are the conditional 1-octanol-water partition coefficients at given solution conditions of the hydroxide complex and of the neutral complexes formed with other monovalent anions X_i^- (excluding OH^-) and α_{R_3SnOH}, and $\alpha_{R_3SnX_i}$ are the fractions of the respective species in the aqueous phase and are calculated as described previously.

K'_{ow} is related to its K_{ow} at infinite dilution (i.e. the reference state) by

$$K'_{ow} = \frac{[TOT]_{org}}{[TOT]_{aq}} = K_{ow} \frac{f_{TOT}\ (aq)}{f_{TOT}\ (org)} \qquad [11]$$

where, f_{TOT} (aq) and f_{TOT} (org) are the activity coefficients of the species in the aqueous and organic phase, respectively. It is assumed that f_{TOT} (org) does not change significantly with electrolyte and TOT concentrations and, therefore, can be set equal to 1 (the effect of ionic strength on the water content of octanol is considered to be negligible).

Therefore,

$$K'_{ow} = \frac{[TOT]_{org}}{[TOT]_{aq}} = K_{ow} f_{TOT}\ (aq) \quad [12]$$

The contribution of the TOT cations (R_3Sn^+), negatively charged complexes with multivalent anions and possible TOT di- or oligomers are neglected. The significance of partitioning of TOT cations can be assumed to be negligible (Arnold et al., 1997). Concentrations of free TOT cations between 0.02 and 2 μM in 1-octanol were predicted for a typical TOT(aq) concentration of 10 μM at pH 4 and 0.1 mM Cl^-(aq). Under the same conditions, TBTCl and TPTCl concentrations of 230 and 70 μM, respectively, in 1-octanol were calculated. The partitioning of TOT cations is not expected to differ significantly in the presence of other counterions. Thus, even at 0.1 mM ionic strength, the partitioning of TOT cations can be assumed to be negligible.

Using the following data, aqueous speciation and D_{ow} of TBT and TPT as a function of pH and Cl^- concentration were predicted using the previous equations.

Table 2.1 Acidity constants, octanol-water partition constants, formation constants and K^s values of some R_3SnX_i species (from Arnold et al., 1997).

	TBT	**TPT**
Acidity constant (pKa) of R_3SnOH	6.25	5.20
log K_{ow} (R_3SnOH)	4.10	3.53
log K_{ow} (R_3SnCl)	4.76	4.19
Formation constant, log K_i(R_3SnCl)	0.6	0.66
K^S (R_3SnOH) in M^{-1} for NaCl	0.61	0.36

CHAPTER 3

RESULTS

Chapter 3: Results

3.1 Tin analysis

3.1.1 *Sn calibration curves using AAS*

Tin is routinely analysed using AAS at two wavelengths, 224.6 and 286.3 nm. For inorganic tin, absorbance readings were higher at 286.3 nm (Fig. 3.1.1) and this wavelength was used for all further analysis. Standard curves ranged up to 1.26 mM Sn. This necessitated the development of other detection systems as a detection limit of less than 1 μM was required for experimental purposes.

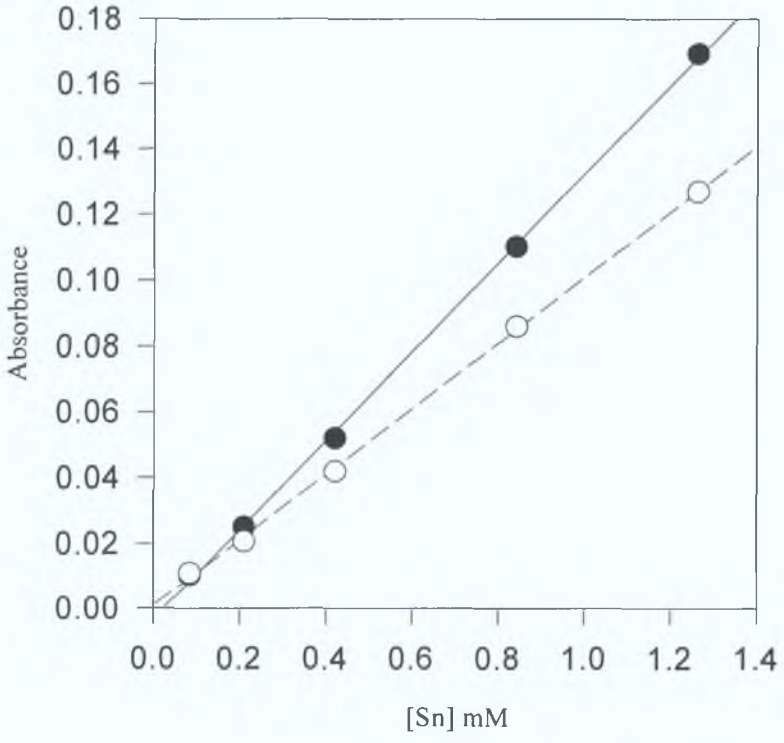

Figure 3.1.1 Sn calibration curves using AAS. Absorbance readings were obtained with wavelength settings of 224.6 nm (open symbols) and 286.3 nm (closed symbols). Standards were prepared in duplicate and each sample was read in triplicate. Mean values are shown and standard deviations were smaller than the symbols used.

3.1.2 *Optimisation of HGAAS*

The oxidant and fuel ratio and HCl and $NaBH_4$ concentrations were optimised for Sn(IV), TBT and TMT analysis using HGAAS. By varying the ratio of air and acetylene, the atomisation cell temperature was varied. At air settings above 6 there was noise in the system and the baseline fluctuated while the flame was found to quench when the fuel was set below 2. No significant difference was found between these ratios so it was decided to use air and acetylene settings of 5 and 2, respectively.

The effect of HCl concentration on TBT detection is shown in Table 3.1.1. Absorbance readings increased with HCl concentration so 1 M HCl was chosen for further analysis. 1% (w/v) $NaBH_4$ was chosen as the optimum reducing agent concentration. At higher concentrations the reaction between $NaBH_4$ and HCl was more vigorous, which resulted in greater error between readings, while lower concentrations were less sensitive (Table 3.1.2).

Table 3.1.1 Effect of acid concentration on determination of 10 μM TBT using 1% $NaBH_4$. Standards were prepared in duplicate and mean values of three determinations are shown.

[HCl] mM	Absorbance ± SD
0.1	0.023±0.002
0.5	0.086±0.005
1	0.093±0.002

Table 3.1.2 Effect of $NaBH_4$ on the determination of 10 μM TBT using 1 M HCl. Standards were prepared in duplicate and mean values of three determinations are shown.

%NaBH (w/v)	Absorbance ± SD
0.5	0.059±0.002
0.8	0.084±0.003
1	0.093±0.002
1.5	0.111±0.01

3.1.3 *HGAAS detection limits and sensitivity*

Typical standard curves achieved using HGAAS under optimum conditions are shown (Fig. 3.1.2). Sensitivity and detection limit for each compound were calculated according to the Perkin Elmer 3100 AAS instruction manual (1982). Sensitivity was defined as the concentration of an element required to produce a signal of 1% absorption (0.0044 absorbance units) and was determined from the standard curves in Fig. 3.1.2. The detection limit was determined as follows: for each compound two concentrations were prepared with one concentration twice that of the other. Both standards were read alternatively, with a blank reading (using 1 M HCl) obtained immediately before and after each. This was repeated 10 times. The two blank readings for each standard were averaged and subtracted from the standard reading. The concentration corresponding to each reading was determined from a suitable standard curve and the mean and standard deviation of each set of ten readings calculated. For the two concentrations, the detection limit was determined from the following equation:

$$\text{Detection Limit} = \frac{\text{Ideal concentration} \times 2_{SD}}{\text{mean concentration}}$$

where, the ideal concentration was the actual prepared value and mean and standard deviation (SD) were calculated for the 10 determinations. The detection limit of each compound was then defined as the mean of these two values. All detection limits and sensitivities were below 1 μM with detection limits decreasing in the order TBT < Sn(IV) < TMT

Table 3.1.3 Detection limit and sensitivity of Sn(IV), TBT and TMT analysis using HGAAS under optimum conditions. Mean detection limits ± SD are shown. Sensitivity was defined as the concentration corresponding to 0.0044 absorbance units and was determined from Fig. 3.1.2.

Compound	Detection limit (μM)	Sensitivity (μM)
Sn(IV)	0.158 ± 0.064	0.081
TBT	0.386 ± 0.087	0.271
TMT	0.040 ± 0.011	0.023

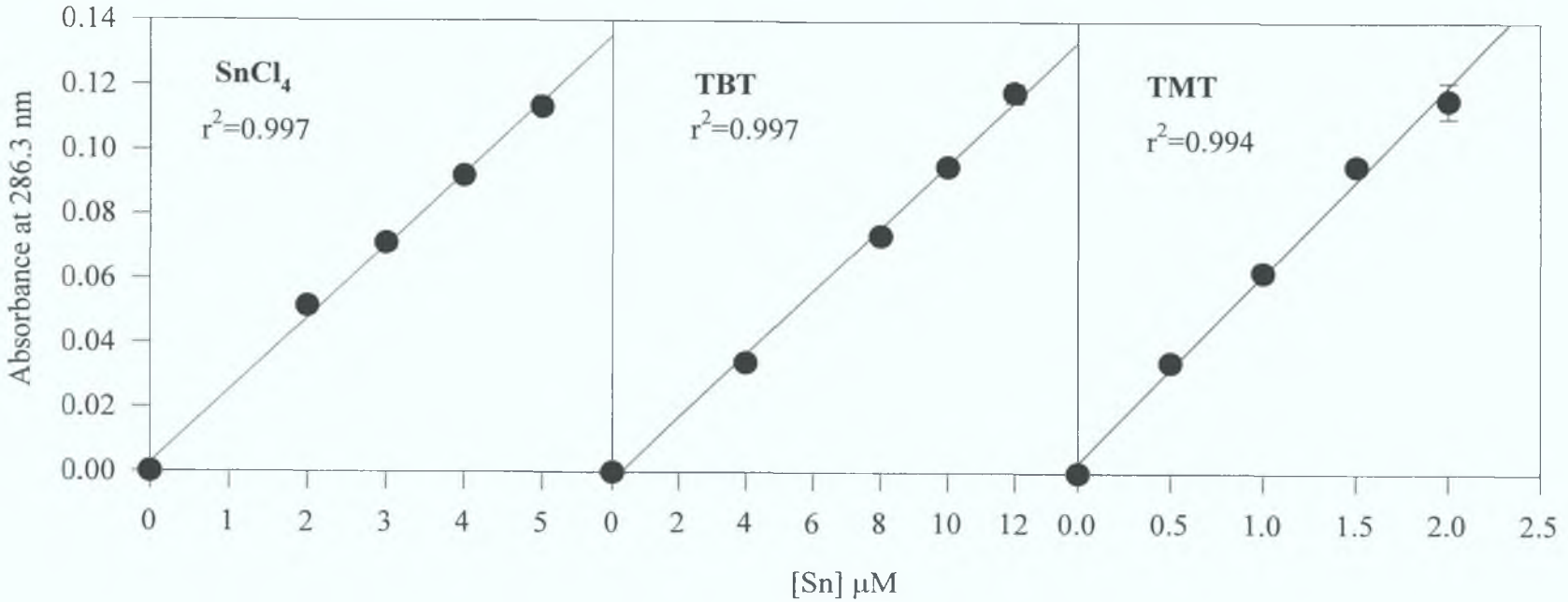

Figure 3.1.2 Sn(IV), TBT and TMT calibration curves using HGAAS. Parameters were set as follows: 1 M HCl at a flowrate of 4.8 ml min^{-1}, 1% $NaBH_4$ at a flowrate of 4.0 ml min^{-1}, wavelength of 286.3 nm and air and acetylene settings of 5 and 2, respectively. Standards were prepared in triplicate and each standard was analysed three times. Mean values ± SD are shown where these exceed the dimensions of the symbols.

3.1.4 *TPT analysis*

TPT is not detected by HGAAS as conversion to hydrides is poor, so differential pulse polarograhy was used for analysis. A typical TPT calibration curve is shown below (Fig. 3.1.3). Standards were prepared in triplicate in 0.16 M NH_4Cl, 40% ethanol and adjusted to pH 2.5. Each standard was analysed three times and TPT peaks were obtained at a half-wave potential of approximately -0.69 V.

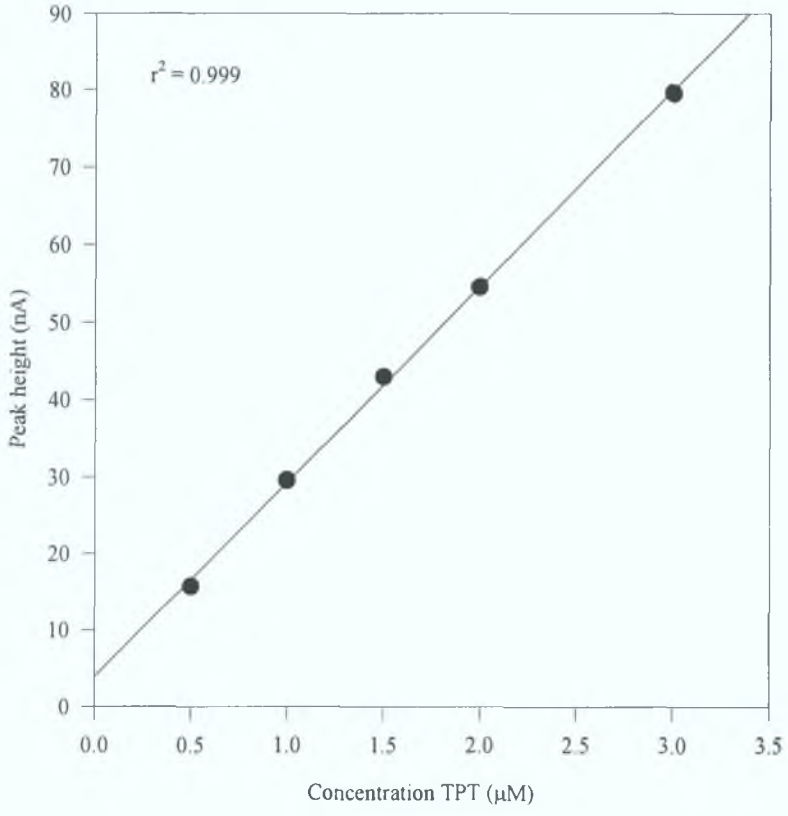

Figure 3.1.3 TPT calibration curve using differential pulse polarograpy. Mean values from triplicate determinations are shown and standard deviations were smaller than the symbols used.

3.1.5 *TBT and TPT Solubility*

The solubility of TBT and TPT in various buffers was assessed by the method described by Inaba et al. (1995). Excess levels of organotin was added to solutions and the soluble concentration analysed. For TBT, approximately 200 μM was added to pH 5.5 and pH 7.5 buffers and 100 μM TBT to 500 mM NaCl, pH 5.5 buffer. 50 μM TPT was added to pH 5.5 buffer and 30 μM to pH 7.5 and 500 mM NaCl, pH 5.5 buffers. An insoluble precipitate was clearly visible in all buffers except for 200 μM TBT at pH 5.5. After mixing for 30 min, samples were centrifuged (2500 x g, 5 min) and the concentration of the supernatants determined. In all cases TPT was less soluble than TBT. Organotin solubility was reduced at pH 7.5 compared to 5.5, while there was also a considerable decrease in solubility in the presence of 500 mM NaCl. To ensure that the following experiments involved soluble organotin concentrations, cells were exposed to maximum concentrations of 100 μM TBT between pH 3.5 and 7.5 and 50 μM TBT in NaCl buffer. For TPT, 30 μM TPT was the maximum exposure concentration between pH 3.5 and 5.5 and 10 μM TPT at pH 7.5. In NaCl, TPT concentrations did not exceed 20 μM. Sn(IV) and TMT were soluble at all required concentrations so the solubility limits were not determined.

Table 3.1.4 TBT and TPT solubility levels in buffered solutions. Excess organotin was added to solution and mixed for 30 min. After centrifugation, the soluble tin concentration was determined. Mean values ± SD from triplicate determinations are shown.

Buffer	**Solubility (μM)**	
	TBT	**TPT**
10 mM MES, pH 5.5	> 200	37.2 ± 2.7
10 mM MES, 500 mM NaCl, pH 5.5	65.7 ± 2.1	24.2 ± 0.5
5 mM PIPES, pH 7.5	149 ± 6.9	12.5 ± 0.3

3.2 Localisation of DPH and TMA-DPH in intact cells and protoplasts

The time required for DPH and TMA-DPH to reach steady state in intact cells and protoplasts was monitored by recording the fluorescence intensity and anisotropy of the probes as they were incorporated into the cells. DPH reached steady state after 30 min in intact cells, and after 10 min in protoplasts (Fig. 3.2.1). In contrast, TMA-DPH was rapidly localised in both intact cells and protoplasts (Fig. 3.2.2). In all cases, both probes remained stable for at least 70 min after incubation.

Anisotropy readings were higher for intact cells compared to protoplasts, indicating that removal of the cell wall resulted in greater membrane fluidity. For DPH, the average steady-state anisotropy between 30 and 70 min was 0.133 for intact cells and 0.096 in protoplasts. For TMA-DPH, average anisotropy readings within this time period were 0.269 and 0.229 for intact cells and protoplasts, respectively. The membrane order parameter, S was also determined. S has a value between 0 and 1 and indicates how ordered a membrane is, with a value of one corresponding to a fully ordered membrane. $S = (r/r_o)^{0.5}$, where r_o is the theoretical limiting anisotropy in the absence of rotational motion and r is the steady state anisotropy measured in the membrane (Gille et al., 1993). For the probes used here, r_o is equal to 0.362 for DPH (Shinitsky and Barenholz, 1978) and 0.395 for TMA-DPH (Gille et al., 1993). Using the steady state values reported above the membrane order parameter for both intact cells and protoplasts was determined (Table 3.2.1). Lower readings were calculated for DPH, indicating that DPH was located within a more fluid region than TMA-DPH.

Table 3.2.1 The membrane order parameter, S, for intact cells and protoplasts of *C. maltosa* determined using DPH and TMA-DPH. Mean S values recorded between 30 and 70 min after incubation from three replicate determinations ± SEM are shown.

	Membrane order parameter, S	
	DPH	**TMA-DPH**
Intact cells	0.606 ± 0.005	0.825 ± 0.01
Protoplasts	0.515 ± 0.003	0.761 ± 0.004

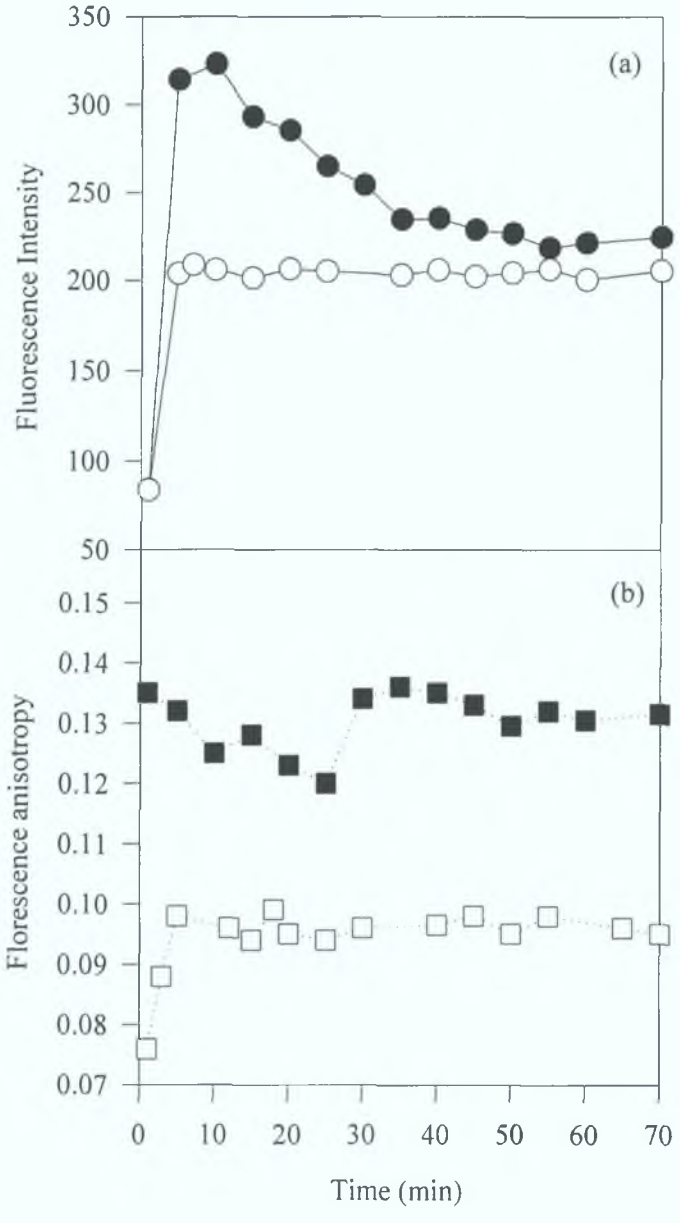

Figure 3.2.1 Incorporation of DPH into intact cells (closed symbols) and protoplasts (open symbols) of *C. maltosa*. Mean values of triplicate determinations of (a) fluorescent intensity and (b) anisotropy readings of DPH recorded in cells at specified time intervals are shown. SEM values were smaller than the dimensions of the symbols.

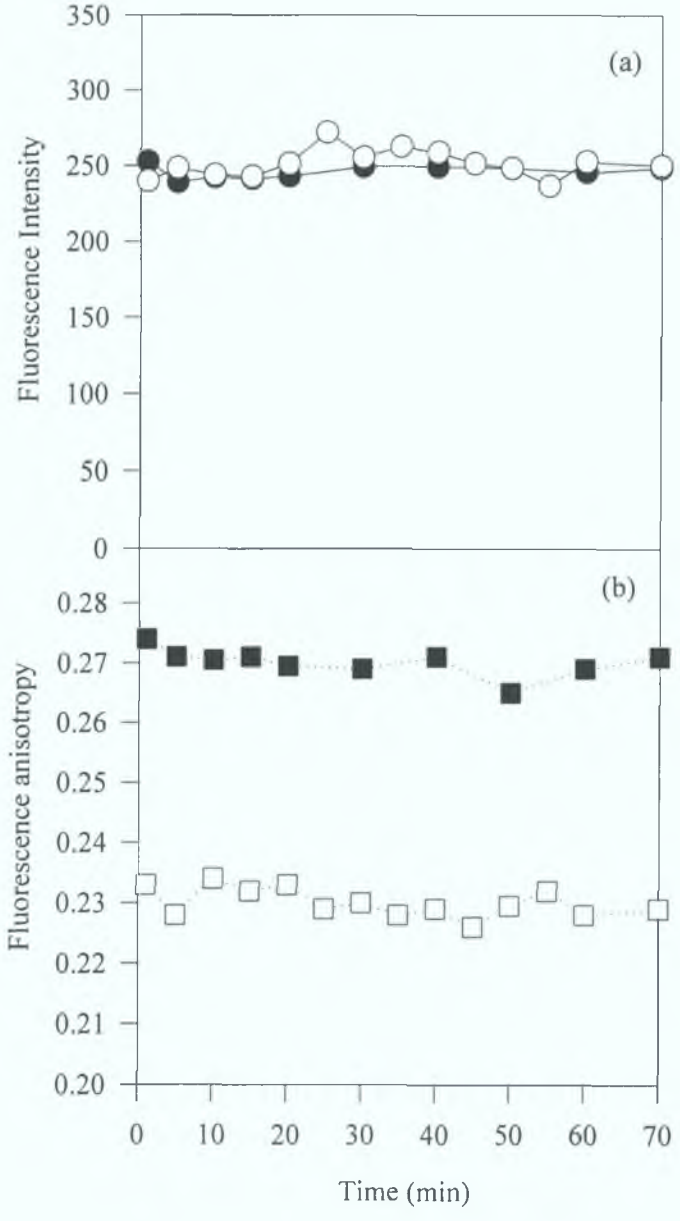

Figure 3.2.2 Incorporation of TMA-DPH into intact cells (closed symbols) and protoplasts (open symbols) of *C. maltosa*. Mean values of triplicate determinations of (a) fluorescent intensity and (b) anisotropy readings of DPH recorded in cells at specified time intervals are shown. SEM values were smaller than the dimensions of the symbols.

The contribution of light scattering to fluorescence measurements was determined as described by Kuhry et al. (1985). Scattering of light from the excitation source to the measured light at the observed wavelength may occur and needs to be taken into account if the fluorescence intensity is low due to the probe concentration. Both intensity and anisotropy are influenced according to:

$$I_{measured} = I_{fluo} + I_{scatter}$$
$$r_{measured} = f r_{fluo} + (1 - f)\, r_{scatter}$$

Where, f (a balanced fluorescence intensity factor) = $I_{fluo} / (I_{fluo} + I_{scatter})$. I_{fluo} and r_{fluo} correspond to the intensity and anisotropy of the labelled sample, respectively. The scattered light intensity ($I_{scatter}$) and anisotropy ($r_{scatter}$) were determined by measuring an unlabelled control under the same conditions as the sample.

At the cell concentrations used in these experiments, $I_{scatter}$ was determined to be less than 10 for both intact cells and protoplasts. As the fluorescence intensity of samples always exceeded 200, light scattering accounted for less than 5% of the measured intensity and the correction for light scattering was not included in any other measurements.

3.3 Comparison of interactions of Sn(IV), TBT and TMT with *C. maltosa* at pH 5.5

3.3.1 *Uptake of Sn(IV), TBT and TMT*

Removal of inorganic tin ($SnCl_4$) and the organotins, TBT and TMT from solution by *C. maltosa* at pH 5.5 was investigated (Fig 3.3.1). Uptake of Sn(IV) and TBT increased with increasing initial solution concentration. TBT uptake levels were similar for both intact cells and protoplasts with most of the organotin being removed from solution at all initial concentrations. Maximum uptake, from an initial concentration of 100 μM TBT, was approximately 90 μmol $(10^{10}$ cells$)^{-1}$. Sn(IV) uptake levels differed for intact cells and protoplasts. Removal of the cell wall resulted in an approximate 2-fold decrease in Sn(IV) uptake capacity. Maximum uptake of Sn(IV) was reduced from 40 μmol Sn $(10^{10}$ cells$)^{-1}$ for intact cells to 19 μmol Sn $(10^{10}$ cells$)^{-1}$ for protoplasts. Removal of TMT from solution at initial concentrations up to 100 μM, was negligible with supernatant concentrations after exposure to cells within 5% of initial levels.

3.3.2 *Effect of Sn(IV), TBT and TMT on cell viability*

The viability of intact cells and protoplasts after contacting with Sn(IV), TBT or TMT was recorded. Viability of intact cells was determined by the standard plate count method, while protoplasts were assessed by a modified methylene blue staining technique. 2% methanol, corresponding to the maximum concentration added from tin stock solutions, had no effect on viability of either intact cells or protoplasts (data not shown).

Uptake of Sn(IV) did not alter the viability of either intact cells or protoplasts (Fig. 3.3.2a). In contrast, cell viability decreased with TBT (Fig. 3.3.2b). At initial concentrations greater than 70 μM, 100% loss in viability of intact cells occurred. TBT toxicity was marginally greater in protoplasts, where 50 μM TBT resulted in total loss of cell viability. However, there was little difference in viability of intact cells and protoplasts after exposure to 20 μM TBT. TMT had no effect on viability of either intact cells or protoplasts (Fig. 3.3.2c).

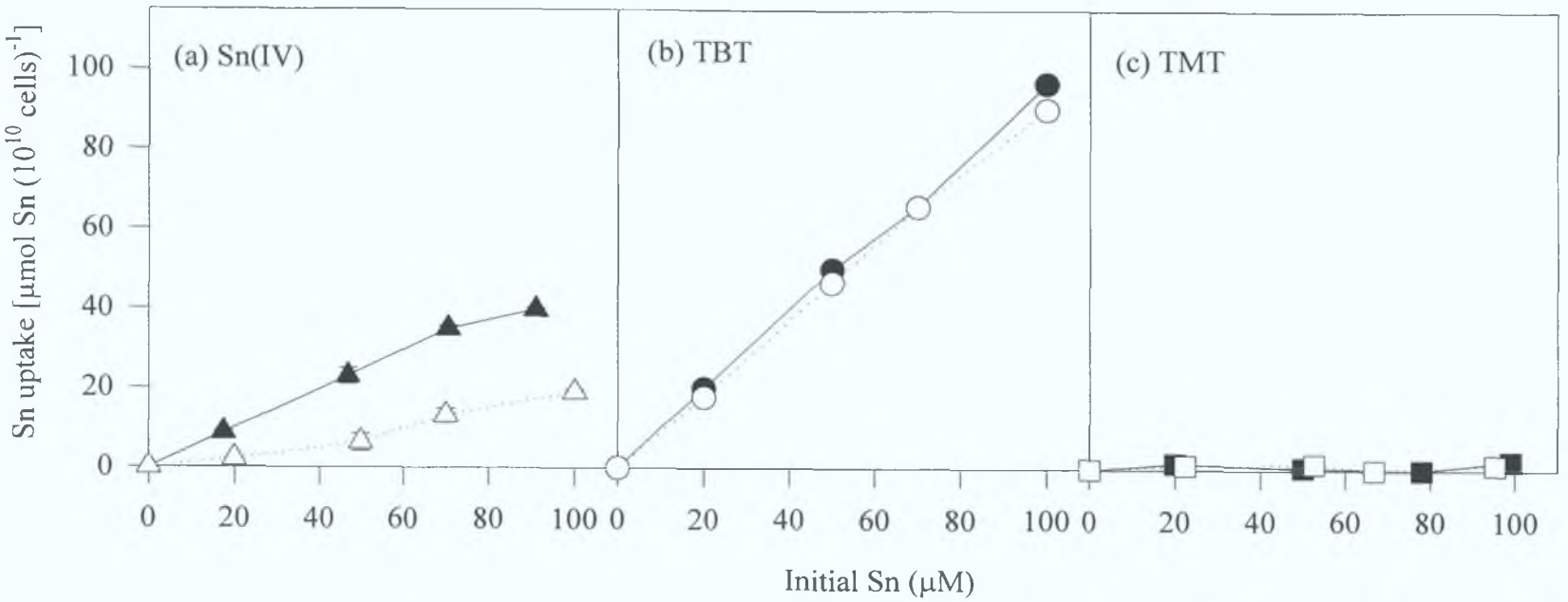

Figure 3.3.1 Uptake of (a) Sn(IV), (b) TBT and (c) TMT by intact cells (closed symbols) and protoplasts (open symbols) of *C. maltosa* at pH 5.5. Mean values ± SEM are shown where these exceed the dimensions of the symbols.

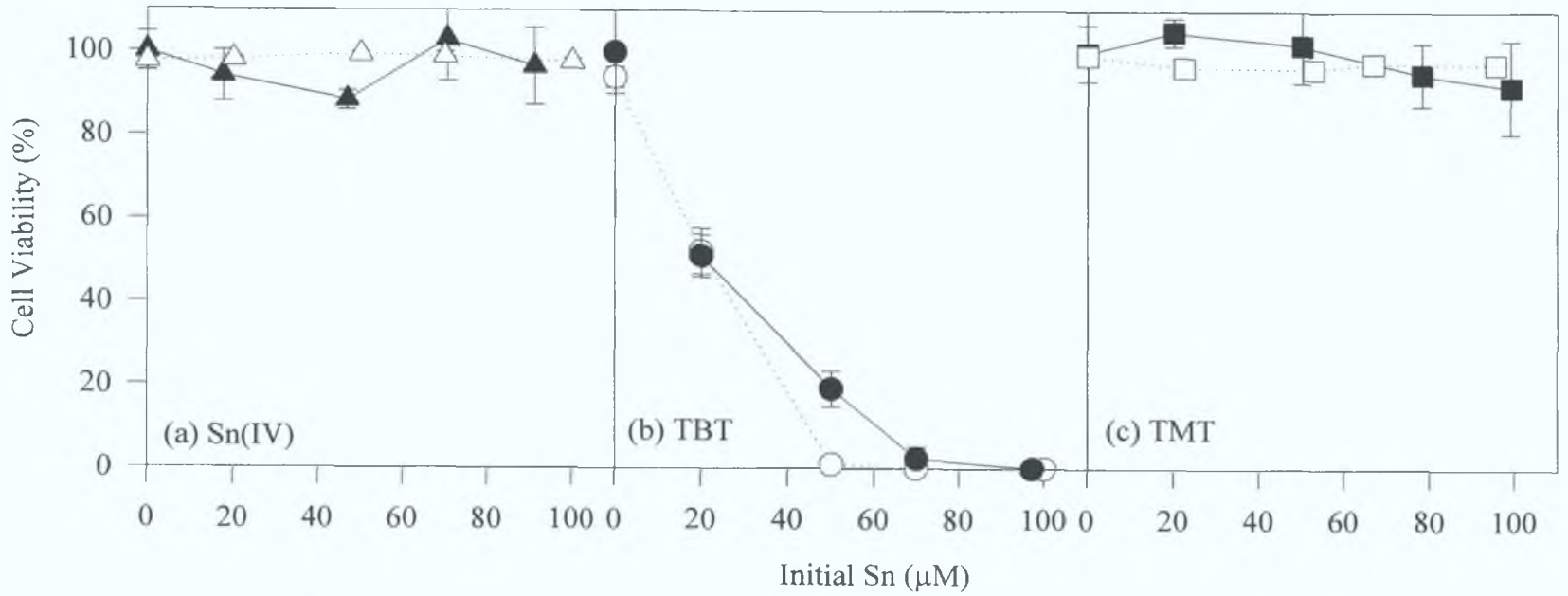

Figure 3.3.2 Viability of intact cells (closed symbols) and protoplasts (open symbols) after exposure to (a) Sn(IV), (b) TBT and (c) TMT. Mean values ± SEM are shown where these exceed the dimension of the symbols.

3.3.3 *Sn(IV)-, TBT- and TMT-induced K^+ release*

The levels of K^+ release from metal-free controls were greater in protoplasts than intact cells (Fig. 3.3.3). 2% methanol, had no effect on K^+ release from cells (data not shown). Inorganic tin, at initial solution concentrations up to 100 μM, did not cause any net change in K^+ release (Fig. 3.3.3a) while exposure to TBT resulted in extensive K^+ leakage from both intact cells and protoplasts (Fig. 3.3.3b). K^+ release from intact cells increased almost linearly with TBT concentration, to a maximum level of 228 μmol K^+ $(10^{10}$ cells$)^{-1}$. In contrast, K^+ release from protoplasts reached an equivalent maximum level at a lower initial concentration of 50 μM TBT. TMT did not affect the levels of extracellular K^+ and levels in solution remained at concentrations equivalent to metal free control values. This is consistent with the absence of viability loss and negligible uptake. No interactions between TMT and cells were apparent at pH 5.5.

3.3.4 *Influence of Sn(IV), TBT and TMT on membrane fluidity*

Prior to experiments, the time required for DPH and TMA-DPH to reach steady-state fluorescence anisotropy in cells was established (Section 3.2). Both probes were restrictively localised in cells during the 30 min tin exposure time. The effects of TBT, TMT and Sn(IV) on the anisotropy of DPH and TMA-DPH were reported as the difference in anisotropy between cells contacted with tin and tin-free controls. 2% methanol had a negligible effect on DPH and TMA-DPH anisotropy.

Uptake of TBT resulted in an increase in the fluorescence anisotropy of DPH in intact cells and protoplasts, reflecting a decrease in membrane fluidity (Fig. 3.3.4). Changes in DPH anisotropy were greater in protoplasts as compared to intact cells at lower initial organotin concentrations. 20 μM TBT had little effect on anisotropy in intact cells, while anisotropy in protoplasts increased by approximately 0.014 units. However, at an initial concentration of 100 μM TBT, anisotropy changes reached similar levels of approximately 0.023 units in both intact cells and protoplasts. Anisotropy of TMA-DPH, which is located at the surface of the cytoplasmic membrane, was not altered by TBT (Fig. 3.3.5). Neither Sn(IV) nor TMT had any effect on fluorescence anisotropy (Fig. 3.3.4 and 3.3.5). The absence of membrane interactions is consistent with the lack of K^+ leakage and change in cell viability.

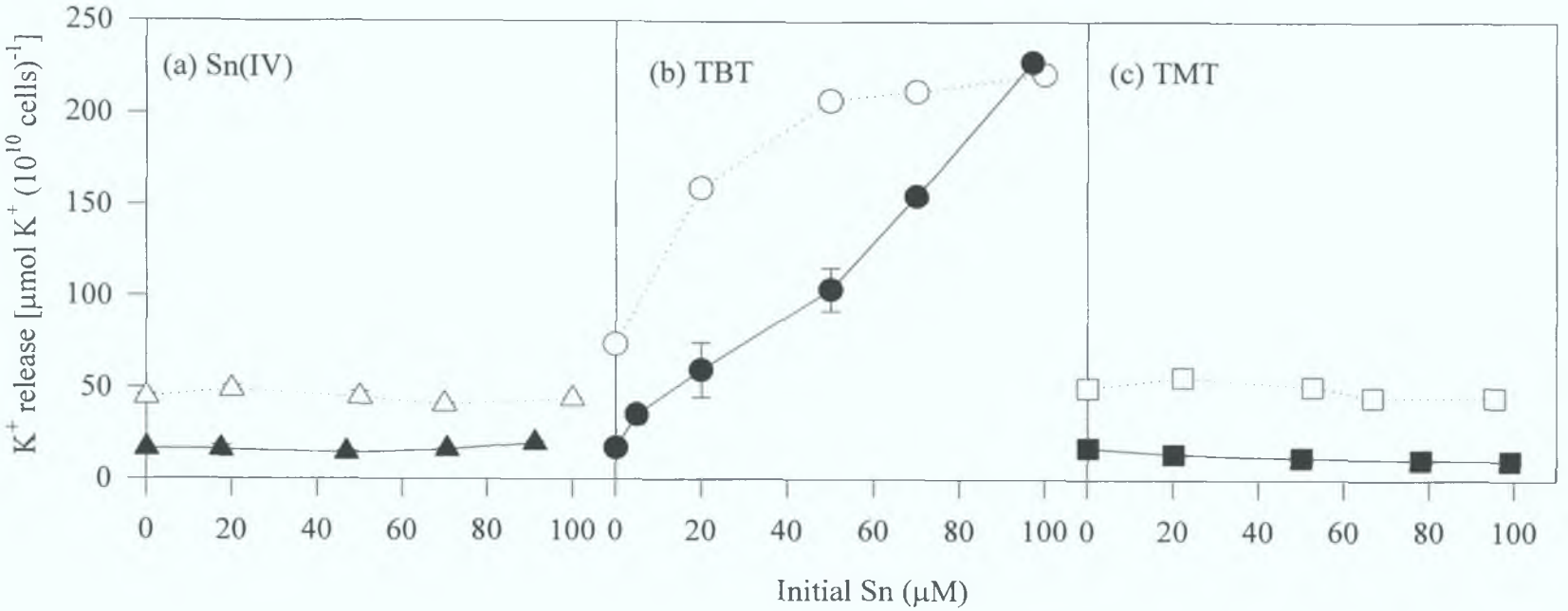

Figure 3.3.3 K^+ release from intact cells (closed symbols) and protoplasts (open symbols) of *C. maltosa* at pH 5.5. Cells were exposed to (a) Sn(IV), (b) TBT and (c) TMT. Mean values ± SEM are shown where these exceed the dimensions of the symbols.

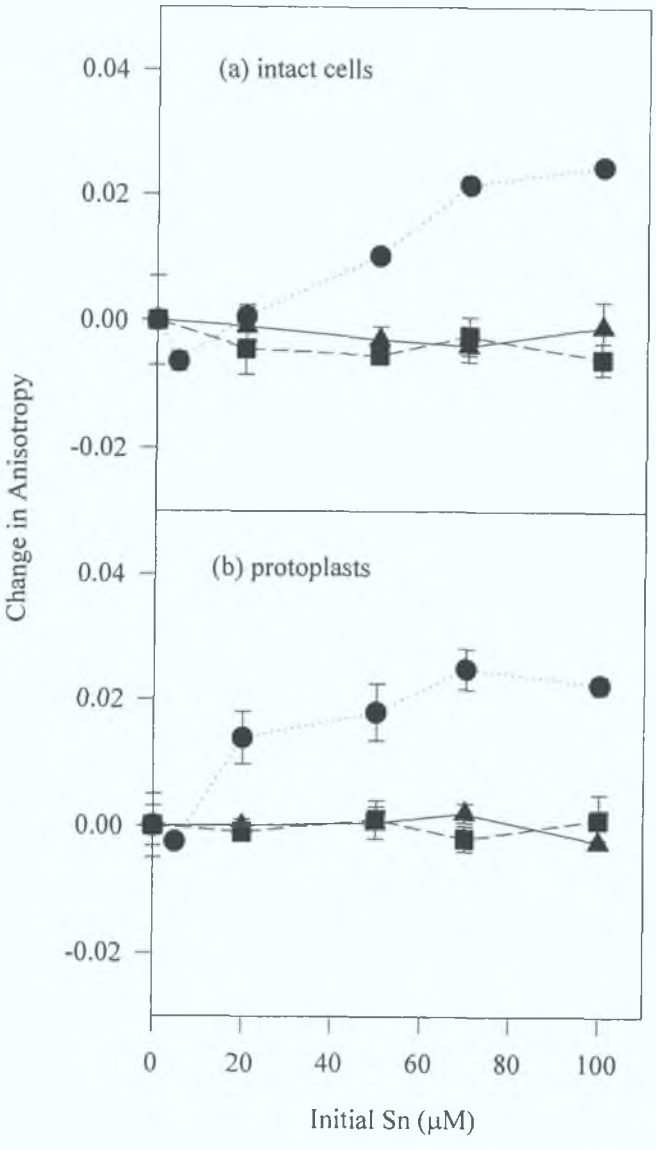

Figure 3.3.4 Influence of Sn(IV) (▲), TBT (●) and TMT (■) and on the fluorescence anisotropy of DPH in (a) intact cells and (b) protoplasts of *C. maltosa* at pH 5.5. Mean values ± SEM are shown where these exceed the dimensions of the symbols.

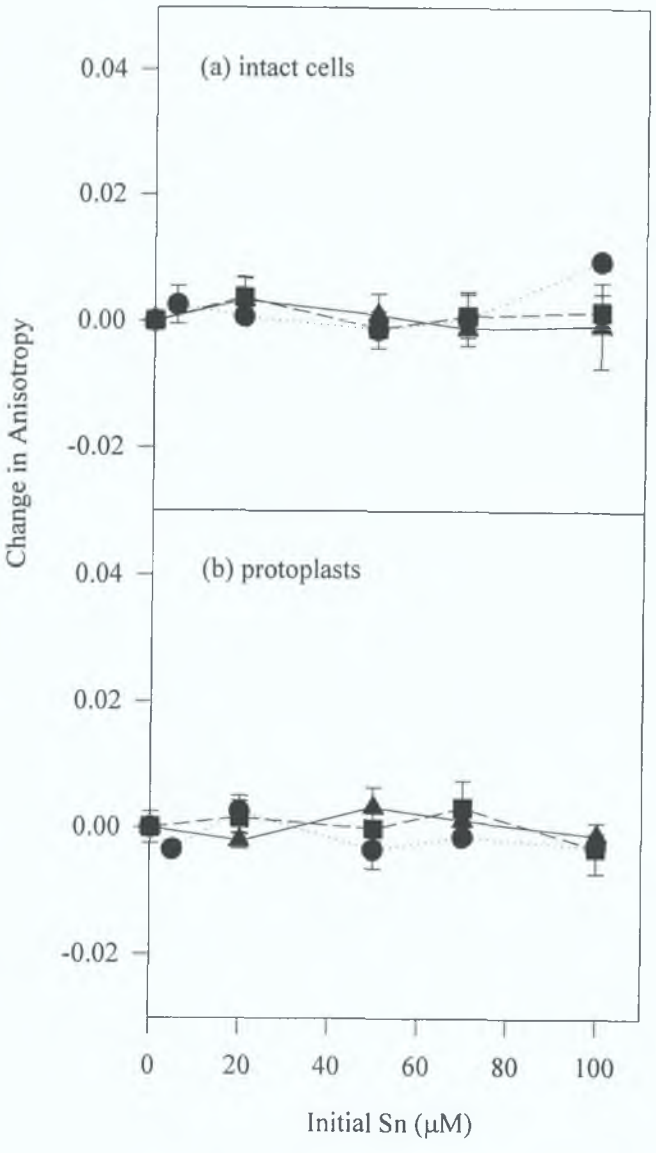

Figure 3.3.5 Influence of Sn(IV) (▲), TBT (●) and TMT (■) and on the fluorescence anisotropy of TMA-DPH in (a) intact cells and (b) protoplasts of *C. maltosa* at pH 5.5. Mean values ± SEM are shown where these exceed the dimensions of the symbols.

3.4 Influence of pH on TMT uptake and toxicity

Uptake of TMT by intact cells was examined between pH 3.5 and pH 7.5 (Fig. 3.4.1). Maximum TMT uptake was 7 μmol $(10^{10}$ cells$)^{-1}$ from an initial concentration of 100 μM at pH 4.5. In all other cases, the levels of TMT remaining after exposure to cells for 30 min were comparable to initial concentrations, indicating the absence of TMT uptake. TMT did not affect the levels of extracellular K^+, which were comparable to those of organotin free controls and there was no significant change in cell viability. TMT did not interact with intact cells and solution pH did not influence results.

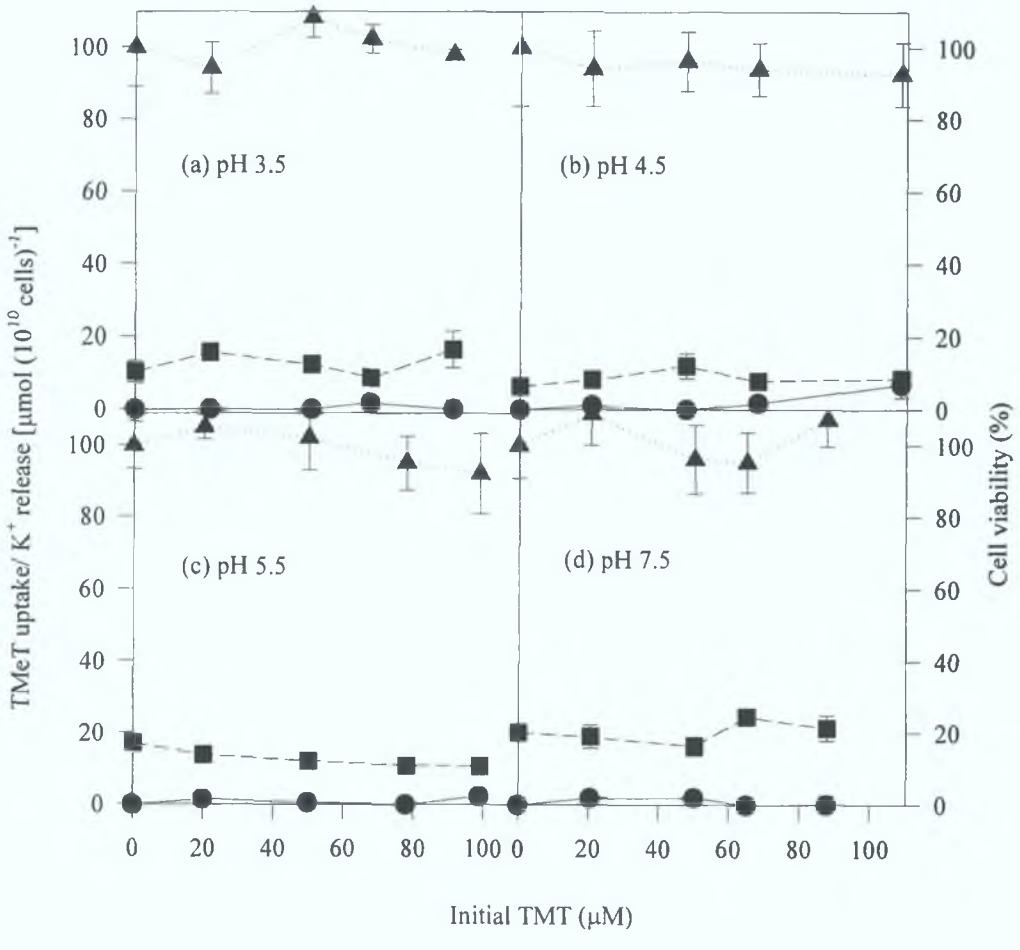

Figure 3.4.1: Influence of pH on TMT uptake and toxicity. TMT uptake (●), K^+ release (■) and % viability (▲) was measured after exposure to TMT at (a) pH 3.5, (b) pH 4.5, (c) pH 5.5 and (d) pH 7.5. Results from duplicate experiments ± SEM are shown.

3.5 Influence of pH on speciation and interactions of TBT and TPT with *C. maltosa*

3.5.1 *Variation in TBT and TPT speciation and D_{ow} with pH*

The fraction of hydrated cation species, $R_3Sn(H_2O)_2^+$, represented here as TBT^+, TPT^+ or R_3Sn^+, and neutral hydroxide species, R_3SnOH, present under various solution conditions was predicted using equations 1-2 in Section 2.5. At pH 3.5, in the absence of other ions, R_3Sn^+ species were dominant, with 0.2% TBT and 2% TPT present as the neutral species, respectively (Fig. 3.5.1). The acidity constant (pK_a) of TPT is approximately one unit below that of TBT so TPT cationic species predominate below pH 5.2, while TBT cationic species are dominant below pH 6.25. At higher pH values, hydroxide species are dominant. Greater than 95% of TPT was calculated to be present as TPTOH above pH 6.5, with 64% of TBT existing as TBTOH, increasing to 95% at pH 7.5.

The octanol-water distribution ratio of organotins also changes with solution composition. An increase in the fraction of neutral species with pH corresponds to greater D_{ow} values (Fig. 3.5.2). At pH 3.5, the D_{ow} of TBT and TPT was calculated as 1.36 and 1.83, respectively. The D_{ow} of TBT increased to a maximum of 4.1 at pH 7, while the D_{ow} of TPT reached a maximum of 3.53 at pH 6. Above these pH values, the D_{ow} ratios remained constant.

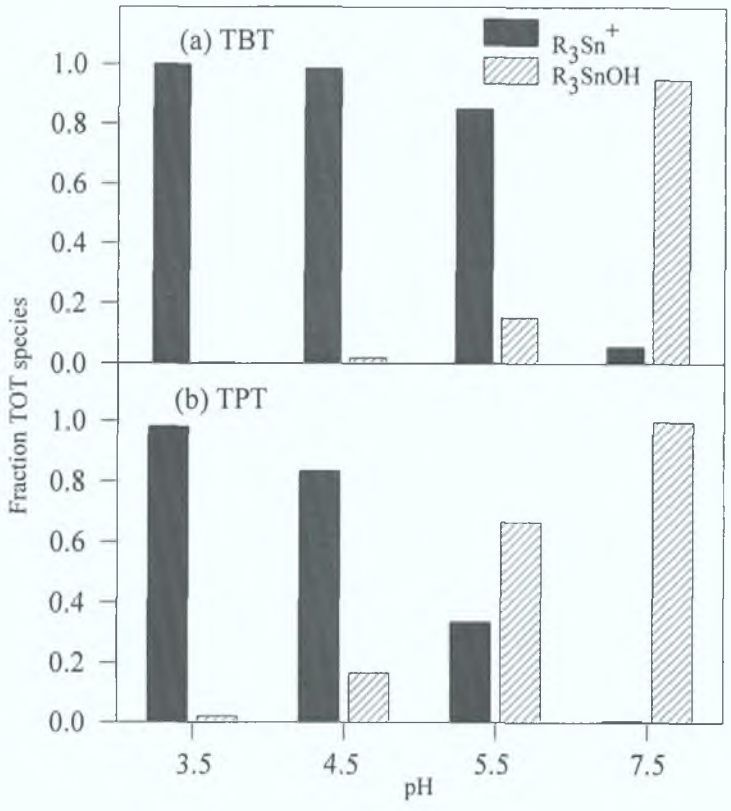

Figure 3.5.1 Variation in (a) TBT and (b) TPT speciation with pH. The predicted species are based solely on solution pH and do not take the presence of other anions into account.

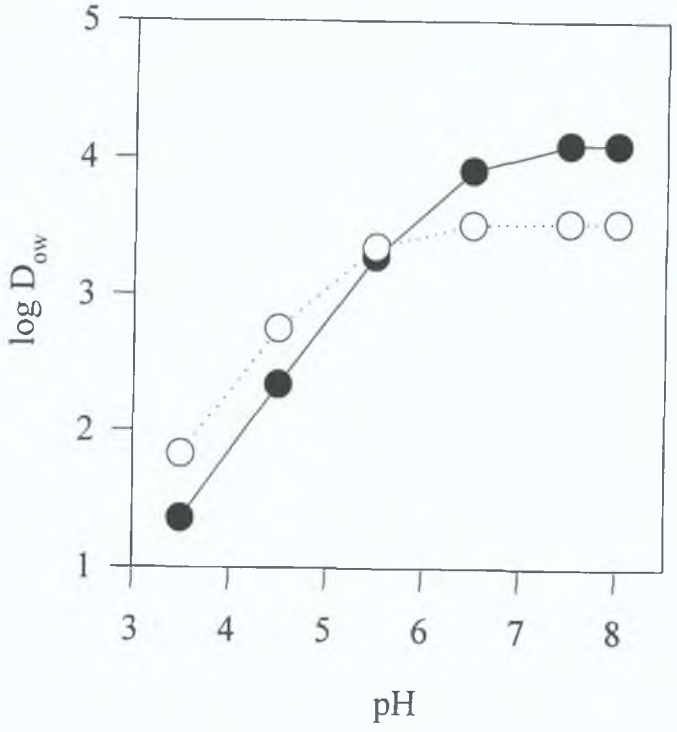

Figure 3.5.2 Dependence of the octanol-water distribution ratio (D_{ow}) of TBT (closed symbols) and TPT (open symbols) on solution pH.

3.5.2 *Influence of pH on the interactions of TBT and TPT with C. maltosa*

Prior to experiments, cell suspensions were washed with either 10 mM MES or 5 mM PIPES buffer, adjusted to the appropriate pH. Comparison of results for cells analysed in the absence of organotin demonstrated that solution pH, between pH 3.5 and 7.5, had little effect on the viability, K^+ leakage or membrane fluidity of cells.

3.5.2.1 Influence of pH on TBT and TPT uptake

There was little difference in TBT uptake between pH 4.5-7.5 (Fig. 3.5.3a). After exposure to initial concentrations up to 100 μM TBT, almost all of the organotin was removed from solution. Maximum uptake levels were 97, 93 and 100 μmol $(10^{10}$ cells$)^{-1}$ at pH 4.5, 5.5 and 7.5 respectively. In contrast, TBT uptake, at all initial concentrations was reduced at pH 3.5. Maximum uptake after exposure to 100 μM TBT was 66 μmol $(10^{10}$ cells$)^{-1}$.

TPT uptake was also lowest at pH 3.5, with a maximum uptake level of 12.3 μmol $(10^{10}$ cells$)^{-1}$ from an initial concentration of 30 μM (Fig. 3.5.3b). There was little difference in uptake at pH 4.5, 5.5 and 7.5 after exposure to 5 and 10 μM TPT. At higher concentrations, uptake was greater at pH 5.5 compared to pH 4.5. Maximum uptake at pH 5.5 was 21.8 μmol $(10^{10}$ cells$)^{-1}$, compared to 15.0 μmol $(10^{10}$ cells$)^{-1}$ at pH 4.5. Due to the reduced solubility of TPT at pH 7.5, 10 μM was the highest concentration examined and no differences in uptake levels compared to pH 4.5 and 5.5 were apparent.

Uptake of TPT was less than TBT in all cases. At initial concentrations of 20 μM TBT, 10.6 μmol $(10^{10}$ cells$)^{-1}$ was removed at pH 3.5, while TBT uptake levels were approximately 20 μmol $(10^{10}$ cells$)^{-1}$ at pH 4.5 and 5.5. In contrast, TPT uptake levels at the corresponding initial concentration were 4.6, 11.4 and 13.8 μmol $(10^{10}$ cells$)^{-1}$ at pH 3.5, 4.5 and 5.5, respectively.

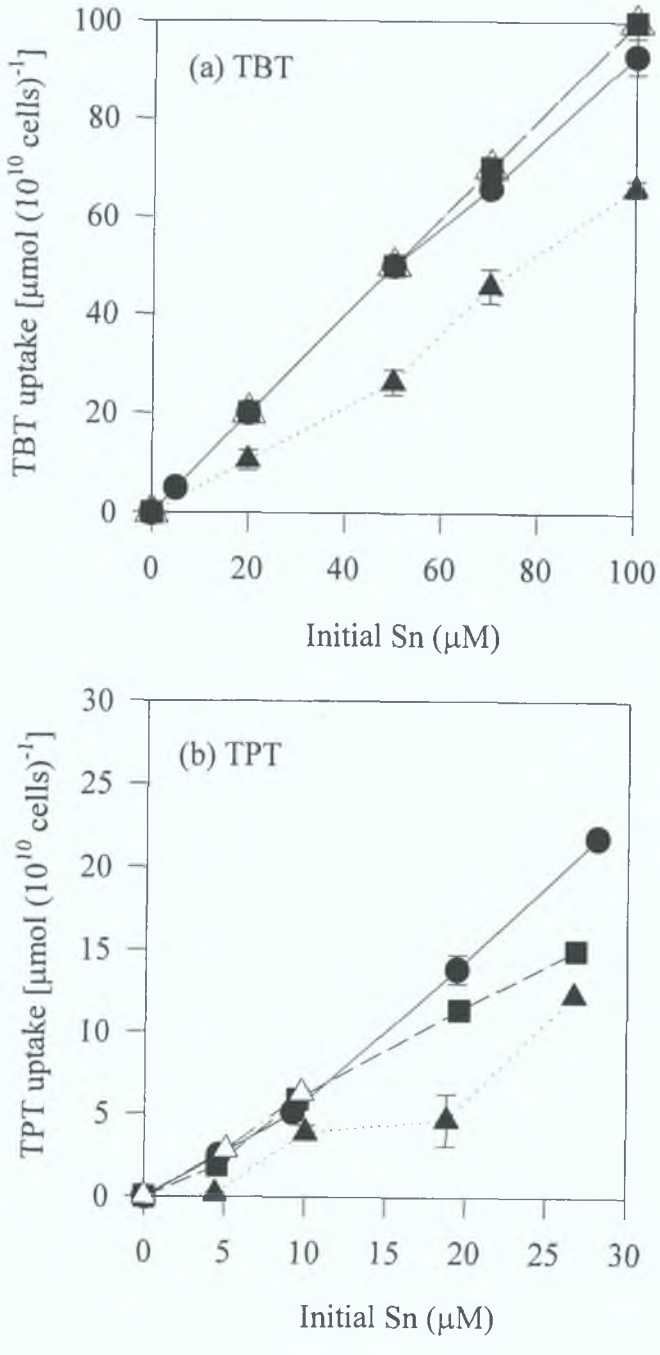

Figure 3.5.3 Influence of pH on the uptake of (a) TBT and (b) TPT. Organotin uptake was measured at pH 3.5 (▲), 4.5 (■), 5.5 (●) and 7.5 (Δ). Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

3.5.2.2 Influence of pH on TBT and TPT toxicity

Organotin-induced cell death increased with external pH (Fig. 3.5.4 and 3.5.5). At the highest TBT concentration examined, cell viability was reduced to 27% at pH 3.5 and 6% at pH 4.5. In contrast, complete loss in cell viability occurred at concentrations greater than 70 and 50 μM TBT at pH 5.5 and 7.5, respectively. At 30 μM TPT, cell viability was reduced to 81, 70 and 62% at pH 3.5, 4.5 and 5.5, respectively. Toxicity of TPT was marginally greater at pH 7.5 compared to 5.5. Exposure to 10 μM TPT, the highest concentration examined at pH 7.5, resulted in 77 and 69 % cell viability at pH 5.5 and 7.5, respectively.

Buffer pH had little effect on K^+ release from cells with levels of approximately 15 – 45 μmol $(10^{10}$ cells$)^{-1}$ occurring in the absence of organotin. TBT-induced K^+ leakage was greatest at pH 5.5 and decreased above and below this pH (Fig. 3.5.4). At pH 3.5, there was very little membrane leakage, with a maximum K^+ level of 48 μmol $(10^{10}$ cells$)^{-1}$ resulting at 100 μM TBT. In the absence of TBT, the external K^+ concentration was 33 μmol $(10^{10}$ cells$)^{-1}$. Maximum K^+ release, after exposure to 100 μM TBT was 139 and 117 μmol $(10^{10}$ cells$)^{-1}$ at pH 4.5 and 7.5, respectively. In contrast, an approximate 2-fold increase in K^+ leakage was apparent at pH 5.5, with 230 μmol K^+ $(10^{10}$ cells$)^{-1}$ released. At lower concentrations, the influence of pH, between 4.5 and 7.5, was not as pronounced. Exposure to 50 μM TBT resulted in K^+ release levels of 90, 104 and 99 μmol $(10^{10}$ cells$)^{-1}$ at pH 4.5, 5.5 and 7.5, respectively. At the same initial TBT concentration, K^+ release only reached 38 μmol $(10^{10}$ cells$)^{-1}$ at pH 3.5.

K^+ leakage in the presence of TPT increased with external pH (Fig. 3.5.5). At pH 3.5, K^+ release levels reached 59 μmol $(10^{10}$ cells$)^{-1}$ after exposure to 30 μM TPT, compared to 45 μmol $(10^{10}$ cells$)^{-1}$ in the absence of organotin. At pH 4.5, exposure to 30 μM TPT resulted in an increase of K^+ leakage from 17 to 51 μmol $(10^{10}$ cells$)^{-1}$. At all TPT concentrations, K^+ release was greater at pH 5.5 compared to 4.5. The maximum K^+ release at pH 5.5 was 132 μmol $(10^{10}$ cells$)^{-1}$, after exposure to 30 μM TPT. K^+ release at pH 7.5 was marginally greater than at pH 5.5. K^+ leakage increased from 41 to 114 μmol $(10^{10}$ cells$)^{-1}$ at pH 7.5 and from 30 to 83 μmol $(10^{10}$ cells$)^{-1}$ at pH 5.5, after exposure to 10 μM TPT.

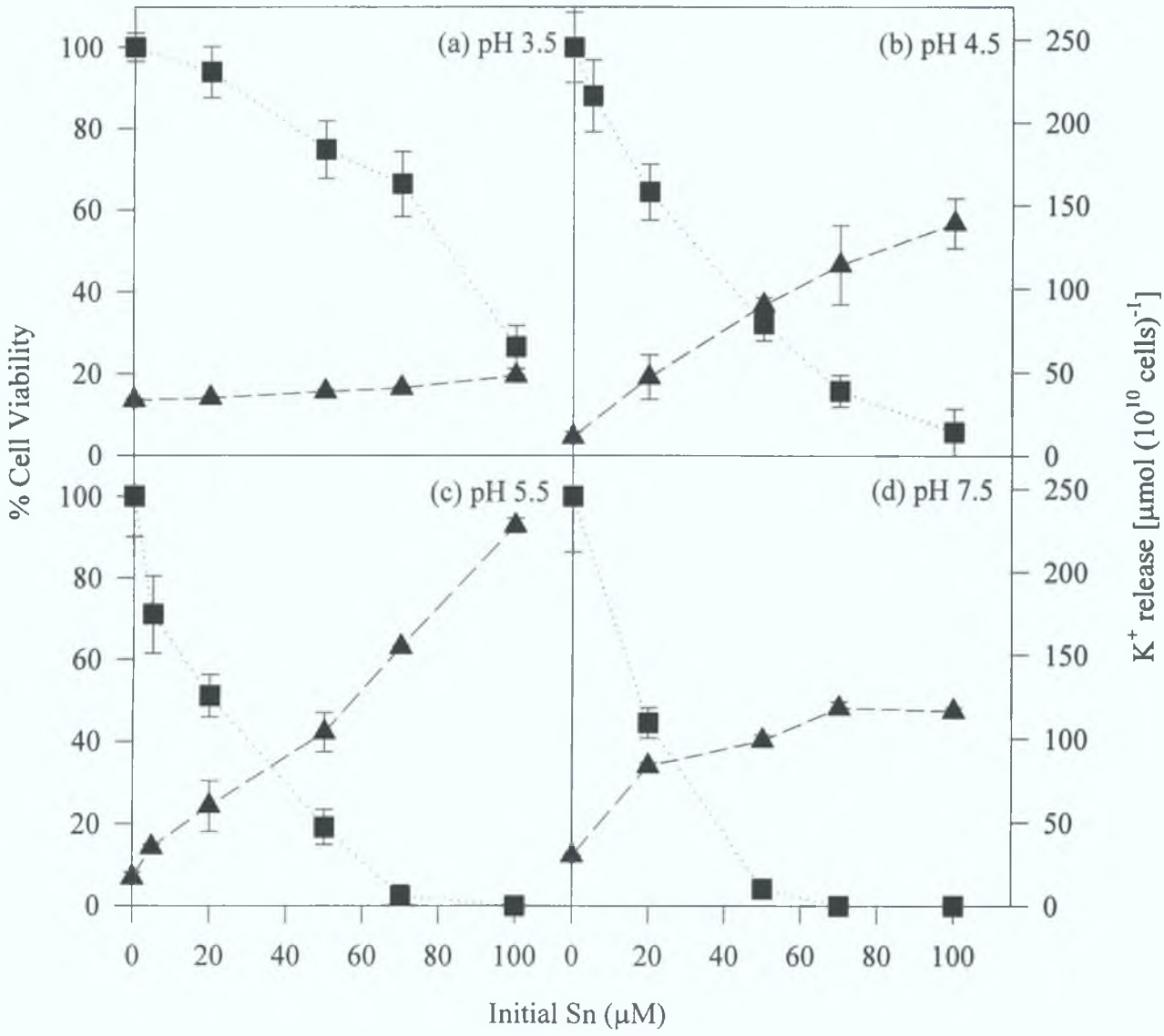

Figure 3.5.4 Influence of pH on the effect of TBT on the cell viability (■) and K^+ release (▲) of *C. maltosa*. Cells were exposed to a range of TBT concentrations in (a) pH 3.5, (b) 4.5, (c) 5.5 and (d) 7.5 buffered solutions. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

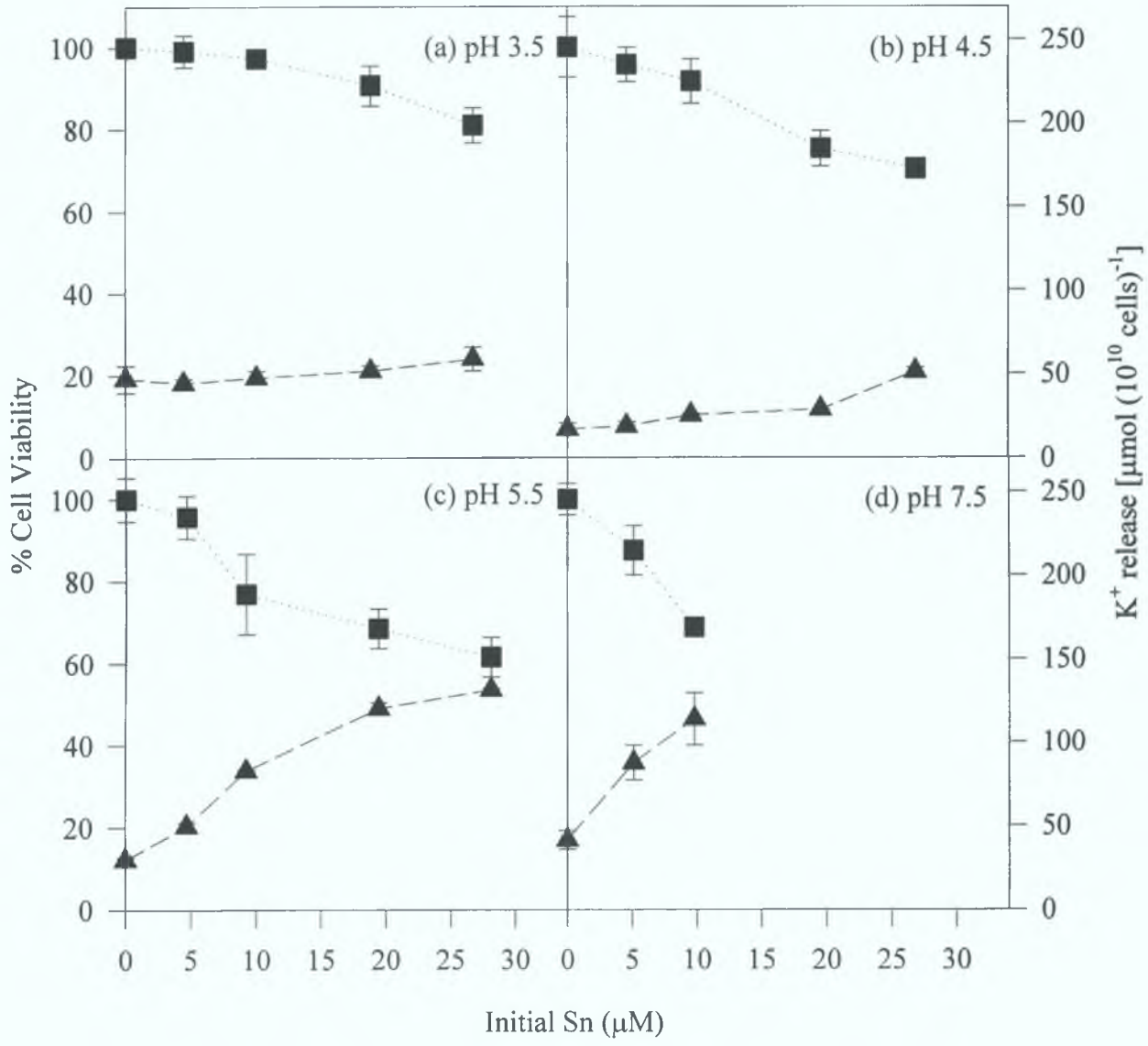

Figure 3.5.5 Influence of pH on the effect of TPT on the cell viability (■) and K^+ release (▲) of *C. maltosa*. Cells were exposed to a range of TPT concentrations in (a) pH 3.5, (b) 4.5, (c) 5.5 and (d) 7.5 buffered solutions. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

Different uptake levels complicated comparisons between TBT and TPT toxicity. Uptake of TPT was less than TBT in all cases. Thus, differences between TBT and TPT toxicity at the same exposure levels may be due to differing uptake levels or actual differences in toxic effects of the compounds after removal from solution. However, at pH 4.5 and 5.5, the effect of the organotins on cell viability were clearly similar at equivalent uptake levels. At pH 4.5, uptake of 15 μmol TPT $(10^{10}$ cells$)^{-1}$, from an initial concentration of 30 μM, resulted in 70% viability. Similarly, exposure to 20 μM TBT caused 64% cell viability, with most of the organotin being removed from solution. At pH 5.5, maximum uptake of TPT to 21.8 μmol $(10^{10}$ cells$)^{-1}$ resulted in 62% viability while removal of 20 μM TBT from solution corresponded to 51% viability. At pH 3.5, however, TPT was more toxic. Uptake of 12.3 μmol TPT $(10^{10}$ cells$)^{-1}$, from an initial concentration of 30 μM, corresponded to 81% viability whereas exposure to 20 μM TBT resulted in an uptake level of 10.6 μmol $(10^{10}$ cells$)^{-1}$ and 94% viability. At pH 7.5, no comparison could be made due to the low solubility of TPT.

K^+ release levels were higher after exposure to TPT compared to TBT at pH 5.5 and 7.5. At pH 5.5, a difference in K^+ leakage of 101 μmol $(10^{10}$ cells$)^{-1}$ before and after exposure to 30 μM TPT occurred at an uptake level of 21.8 μmol TPT $(10^{10}$ cells$)^{-1}$. In comparison, K^+ release increased from 17 to 60 μmol $(10^{10}$ cells$)^{-1}$ after exposure to 20 μM TBT. TBT-induced K^+ release was reduced at pH 7.5 whereas TPT had a greater effect. K^+ release levels increased from 41 to 114 μmol $(10^{10}$ cells$)^{-1}$ and 30 to 84 μmol $(10^{10}$ cells$)^{-1}$ after exposure to 10 μM TPT and 20 μM TBT, respectively. K^+ leakage was reduced at pH 4.5 with TBT and TPT causing similar effects at equivalent uptake levels. Uptake of 15 μmol TPT $(10^{10}$ cells$)^{-1}$ resulted in a difference in K^+ leakage of 34 μmol $(10^{10}$ cells$)^{-1}$ while an identical level occurred after exposure to 20 μM TBT. There was no discernible difference at pH 3.5, with very little membrane leakage at all concentrations examined.

3.5.2.3 Influence of pH on the effects of TBT and TPT on membrane fluidity

The effects of TBT and TPT on the anisotropy of DPH and TMA-DPH were dependent on pH (Fig. 3.5.6 and Fig. 3.5.7). At pH 3.5 and 7.5, at concentrations up to 100 μM, TBT had no effect on membrane fluidity. There was negligible difference between DPH or TMA-DPH anisotropy, in the presence or absence of TBT. TBT altered membrane fluidity to different degrees at pH 4.5 and 5.5. At pH 4.5, TMA-DPH anisotropy decreased with a maximum change of 0.029 units at 50 μM TBT, while DPH anisotropy was unaltered. This indicated that the fluidity of the hydrophilic surface of the cytoplasmic membrane increased in the presence of TBT. In contrast, DPH anisotropy values increased with TBT concentration at pH 5.5, while TMA-DPH anisotropy was unaltered. Change in DPH anisotropy increased to a maximum of 0.024 units at 100 μM TBT. This reflected a decrease in the fluidity of the hydrophobic core of membrane lipids.

There was no change in either DPH or TMA-DPH anisotropy after exposure to TPT at pH 3.5 and 4.5 (Fig. 3.5.7). At pH 5.5, DPH anisotropy increased with organotin concentration, to a maximum change of 0.042 units at 20 μM TPT. There was no change in TMA-DPH anisotropy, indicating that TPT only affected the fluidity of the inner core of membrane lipids. A similar effect was observed at pH 7.5, with DPH anisotropy altered to the same degree. After exposure to 10 μM TPT, the anisotropy of DPH increased by 0.026 units at pH 5.5 and 7.5.

Comparing Fig 3.5.6 and 3.5.7 shows that TBT and TPT have different effects on membrane fluidity. At pH 4.5, TBT caused a decrease in TMA-DPH anisotropy, while TPT had no discernible effect. However, changes in TMA-DPH anisotropy only occurred after exposure to 20 μM TBT, where most of the TBT was removed from solution, while maximum TPT uptake, from an initial concentration of 30 μM, was to a level of 15 μmol $(10^{10}$ cells$)^{-1}$. These trends are similar to those for K^+ leakage, where similar low K^+ release levels occurred after exposure to 20 μM TBT and 30 μM TPT and greater membrane-damaging effects were associated with higher TBT concentrations. Both organotins had a similar effect at pH 5.5, with changes in DPH anisotropy associated with an overall decrease in fluidity at the hydrophobic core of membrane lipids. However, TPT altered DPH anisotropy to a greater extent. At 20 μM

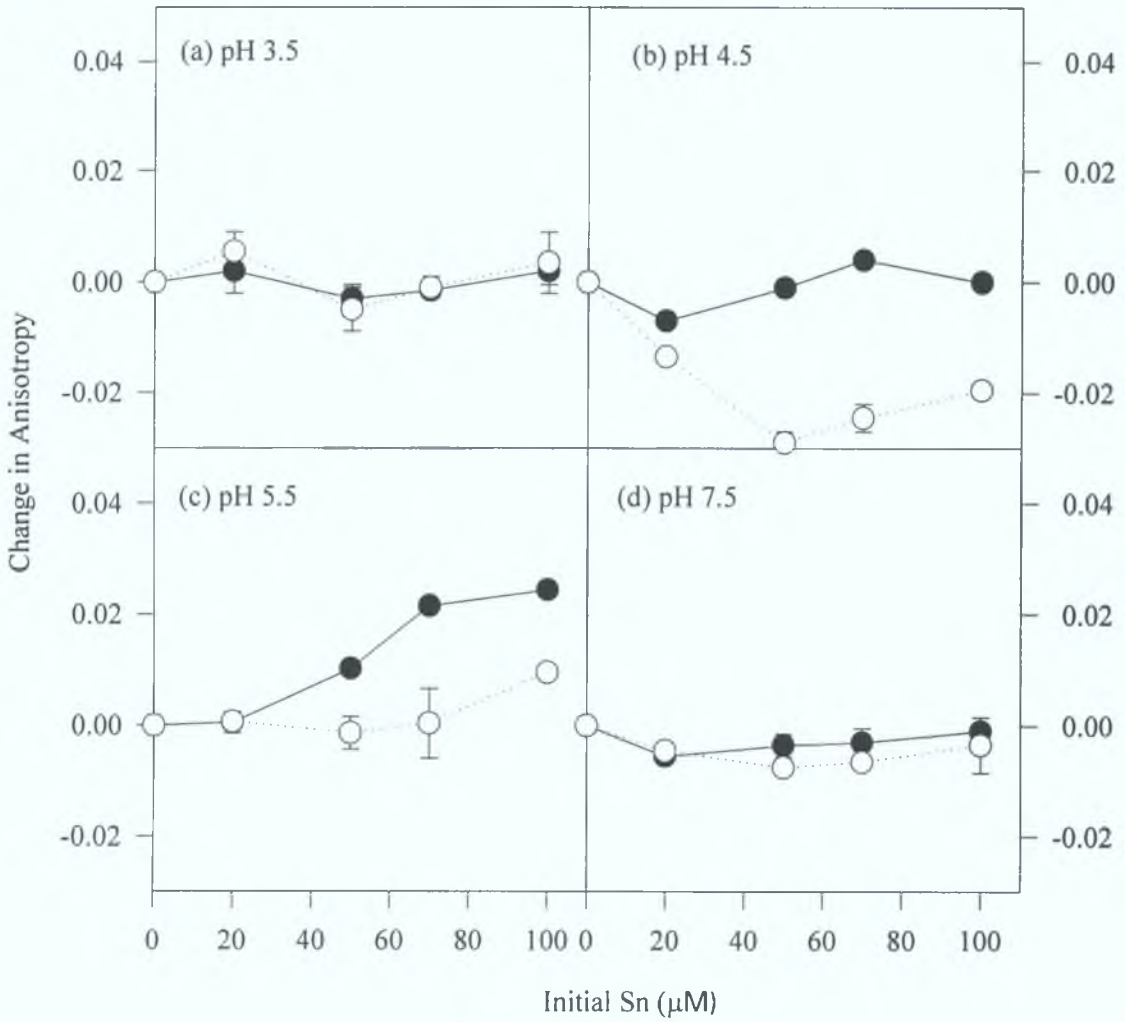

Figure 3.5.6 Influence of pH on the effect of TBT on the membrane fluidity of *C. maltosa*. Cells were exposed to TBT at (a) pH 3.5, (b) 4.5, (c) 5.5 and (d) 7.5 and the effects on anisotropy of either DPH (closed symbols) and TMA-DPH (open symbols) was monitored. Change in anisotropy was calculated as the difference between the anisotropy of cells in the presence and absence of organotin. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

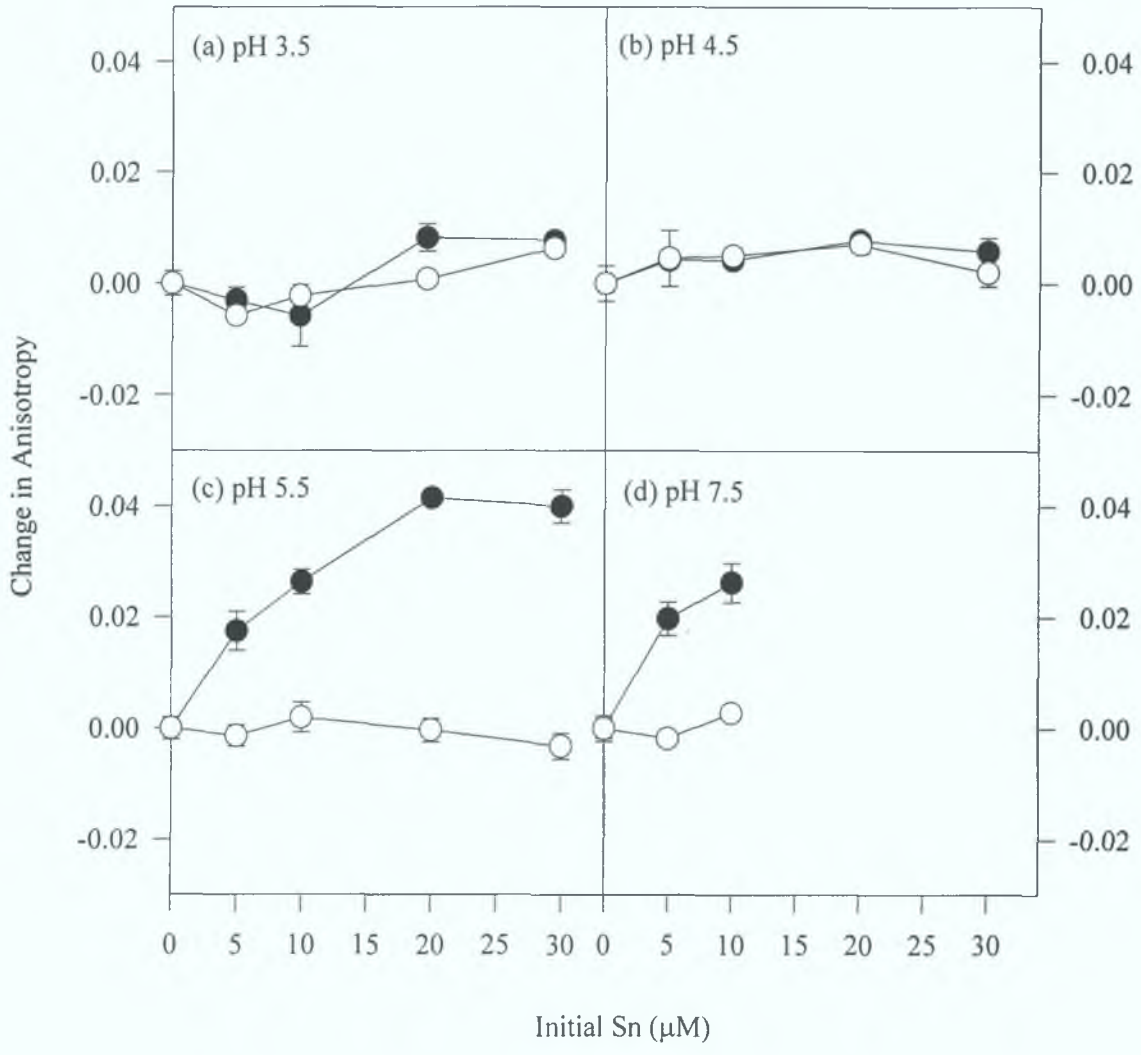

Figure 3.5.7 Influence of pH on the effect of TPT on the membrane fluidity of *C. maltosa*. Cells were exposed to TPT at (a) pH 3.5, (b) 4.5, (c) 5.5 and (d) 7.5 and the effects on anisotropy of either DPH (closed symbols) and TMA-DPH (open symbols) was monitored. Change in anisotropy was calculated as the difference between the anisotropy of cells in the presence and absence of organotin. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

TPT, DPH anisotropy increased by 0.042 units, while TBT only had an effect above this concentration. This too was consistent with K^+ leakage results where TPT uptake resulted in greater release levels than TBT. TPT-induced changes in membrane fluidity were similar at pH 7.5 compared to 5.5 which corresponded with K^+ release levels. However, at pH 7.5, TBT had no effect on membrane fluidity. This was also in agreement with reduced TBT-induced K^+ leakage at pH 7.5 compared to 5.5. At pH 3.5, results were similar, with negligible membrane-damaging effects occurring.

3.5.3 *Relationship between organotin toxicity, pH and D_{ow}*

The relationship between pH and organotin toxicity was investigated further (Fig. 3.5.8 and 3.5.9). Cytotoxicity was expressed as organotin concentrations resulting in 20 and 50% inhibition of cell viability, for TBT and TPT, respectively. Organotin toxicity increased between pH 3.5 and 5.5. There was a 3-4 fold difference in TBT IC_{50} and TPT IC_{20} values within this pH range (Fig. 3.5.8a and 3.5.9a). Toxicity at pH 7.5 was similar to that at pH 5.5 with TBT IC_{50} values of 20.4 and 18 μM and 8.25 and 6.9 μM for TPT at pH 5.5 and 7.5, respectively.

Increased cell toxicity corresponded to an increase in the presence of neutral R_3SnOH species. The influence of compound lipophilicity, expressed as D_{ow} values on cytotoxicity was assessed. There was a clear correlation between D_{ow} and cell death. The correlation was better for TPT than TBT with linear regression coefficients of 0.983 and 0.811, respectively. The toxicity of TBT at pH 5.5 was very similar to 7.5, even though there was only 15% neutral species present compared to 95% at the higher pH.

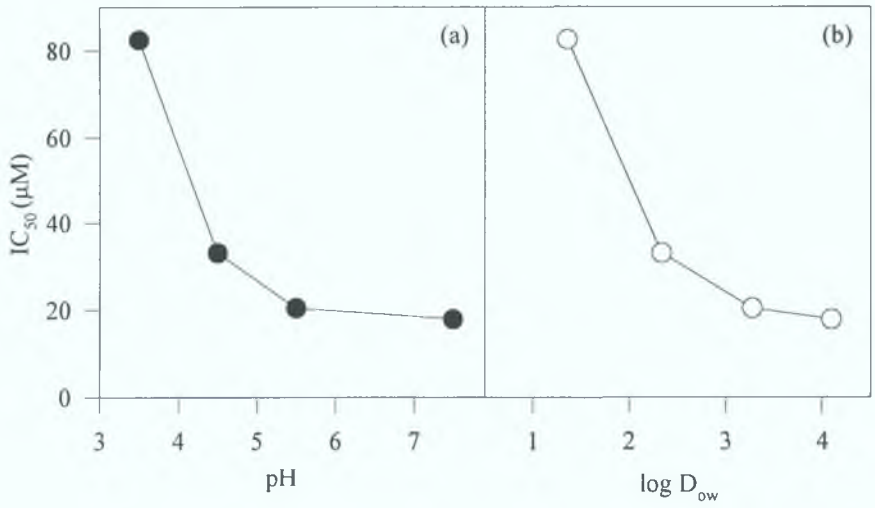

Figure 3.5.8 Variation in IC_{50} of TBT with (a) pH, (b) D_{ow}. IC_{50} were calculated from Fig. 3.5.4.

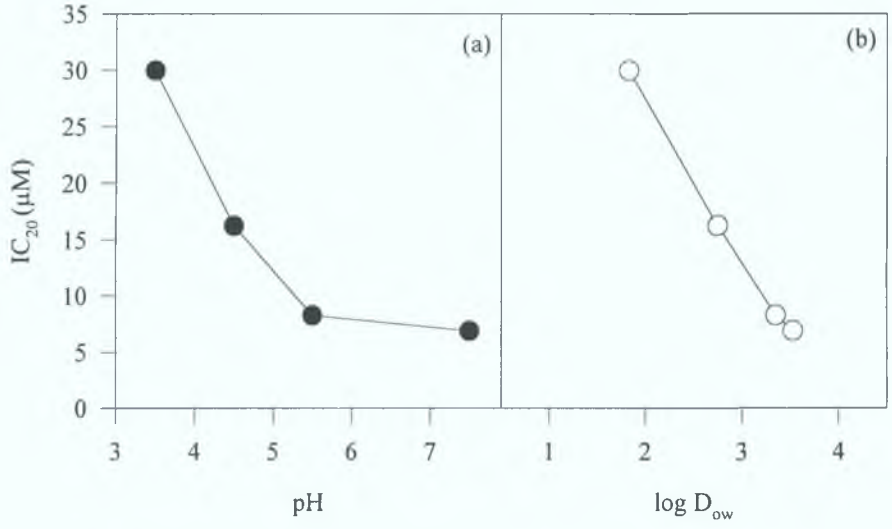

Figure 3.5.9 Variation in IC_{20} of TPT with (a) pH, (b) D_{ow}. IC_{20} were calculated from Fig. 3.5.5.

3.6 Influence of NaCl on speciation and interactions of TBT and TPT with *C. maltosa* at pH 5.5

3.6.1 *Variation in TBT and TPT speciation and D_{ow} with NaCl*

The fraction of R_3Sn^+, R_3SnOH and R_3SnCl species present in aqueous solution at pH 5.5 was predicted between 0 and 500 mM NaCl using the equations in Section 2.5.1. The presence of Cl^- ions resulted in the formation of R_3SnCl species in solution (Fig. 3.6.1). At pH 5.5, in the absence of NaCl, TBT consisted of 85% TBT^+ and 15% TBTOH, while 33 % of TPT was present as TPT^+, with 67% TPTOH. In 500 mM NaCl, the fractions of TBT^+ and TBTOH were reduced to 62 and 4%, respectively, with 34% of TBT present as TBTCl. 35% of TPT was calculated to be present as TPTCl, with 36% TPT^+ and 29% TPTOH. For TPT, the total of neutral TPT species remained relatively constant (67-63%) over the NaCl concentration range with TPTCl being formed in place of TPTOH. For TBT, both the fractions of total neutral and chloride species increased with NaCl, with 15 and 38% neutral species present in 0 and 500 mM NaCl, respectively. In the following experiments, cells were exposed to organotins at pH 5.5. This pH was chosen to allow a broad range of organotin species to be examined with increasing NaCl concentration.

The D_{ow} values of TBT and TPT increased with NaCl concentration (Fig. 3.6.2). This corresponded to the increasing presence of R_3SnCl species, which is more lipophilic than R_3SnOH. At pH 5.5, the D_{ow} of TBT was 3.28 and TPT was 3.35, in the absence of NaCl, increasing to 4.6 and 3.94, respectively, in 500 mM NaCl.

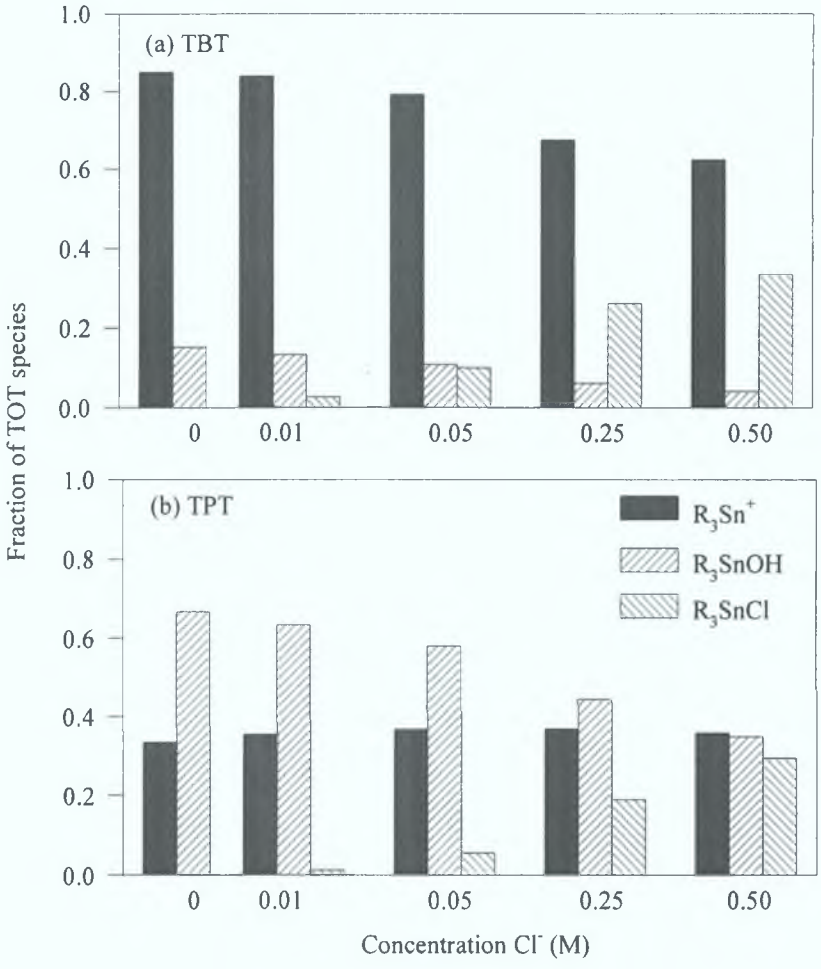

Figure 3.6.1 Effect of Cl^- concentration on the speciation of (a) TBT and (b) TPT at pH 5.5.

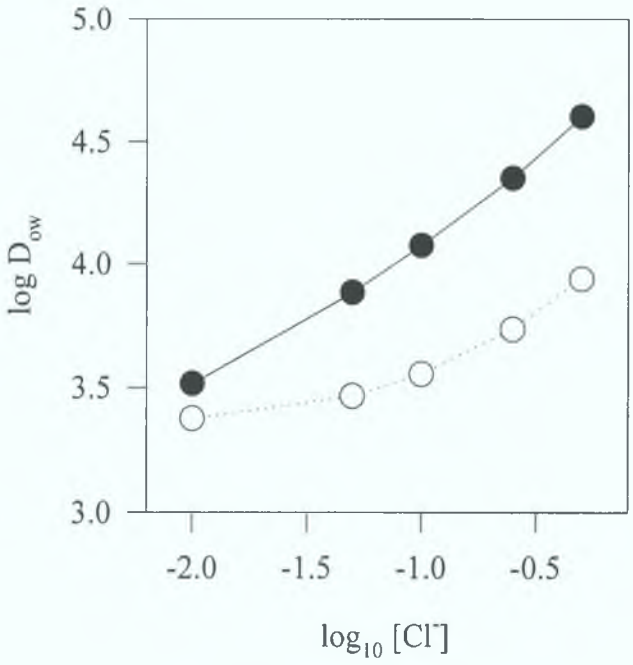

Figure 3.6.2 Variation in D_{ow} values of TBT (closed symbols) and TPT (open symbols) with Cl^- concentration (M).

3.6.2 *Influence of NaCl on the interactions of TBT and TPT with C. maltosa*

To allow the effects of NaCl on *C. maltosa* to be determined, cell suspensions were pre-washed with 10 mM MES, pH 5.5 buffer and then equilibrated with buffer for 30 min before the addition of NaCl. In subsequent experiments, cells were pre-washed with buffer containing the corresponding concentration of NaCl and then equilibrated with this buffer before exposure to organotin compounds. This allowed the effects of NaCl on organotin-yeast interactions to be determined without having to take any NaCl-related cell changes into account.

3.6.2.1 Interactions of NaCl with C. maltosa

NaCl, at initial concentrations up to 500 mM, had no effect on viability of *C. maltosa* after incubating for 30 min at pH 5.5 (Fig. 3.6.3). In contrast, K^+ release increased with NaCl concentration, to a maximum of 130 μmol K^+ $(10^{10}$ cells$)^{-1}$ in 500 mM NaCl. Na^+ uptake was minimal, with less than 5% of the initial concentrations being removed from solution.

The presence of NaCl had no effect on the membrane fluidity of *C. maltosa* (Table 3.6.1). There was no significant change in anisotropy of either DPH or TMA-DPH after 30 min incubation in 50 or 500 mM NaCl, with all values within 2% of the control samples.

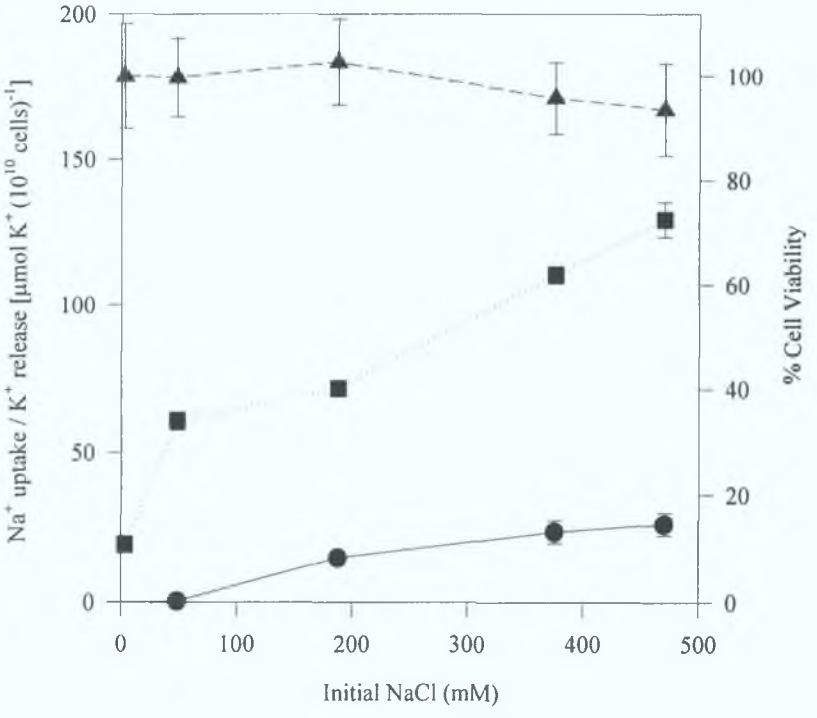

Figure 3.6.3 Uptake and toxicity of NaCl in 10 mM MES, pH 5.5 buffer. Na^+ uptake (●), K^+ release (■) and cell viability (▲) was recorded after exposure for 30 min. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

[NaCl]mM	Anisotropy ± SEM	
	DPH	TMA-DPH
0	0.131 ± 0.002	0.267 ± 0.002
50	0.128 ± 0.001	0.266 ± 0.002
500	0.130 ± 0.004	0.271 ± 0.003

Table 3.6.1 Effect of 50 and 500 mM NaCl on membrane fluidity of *C. maltosa*. The anisotropy of DPH and TMA-DPH was monitored after exposure to 50 and 500 mM NaCl for 30 min. Mean values ± SEM from triplicate determinations are shown.

3.6.2.2 Influence of NaCl on the uptake and toxicity of TBT and TPT

Organotin toxicity, as reported by cell viability and K^+ release, increased in the presence of NaCl. For TBT, this was not due to changes in organotin uptake levels. NaCl had no effect on TBT uptake with most of the organotin removed from solution at all initial concentrations. The TBT concentration in supernatants after exposure to cells was less than 2 μM, with TBT only being detected in samples which had initial exposure concentrations above 50 μM. There was a marginal increase in TPT uptake with NaCl concentration (Fig. 3.6.4). After exposure to 20μM TPT, uptake levels of 13.8, 16.5, 17.1 and 17.2 μmol $(10^{10}$ cells$)^{-1}$ were recorded in 1, 50, 250 and 500 mM NaCl, respectively.

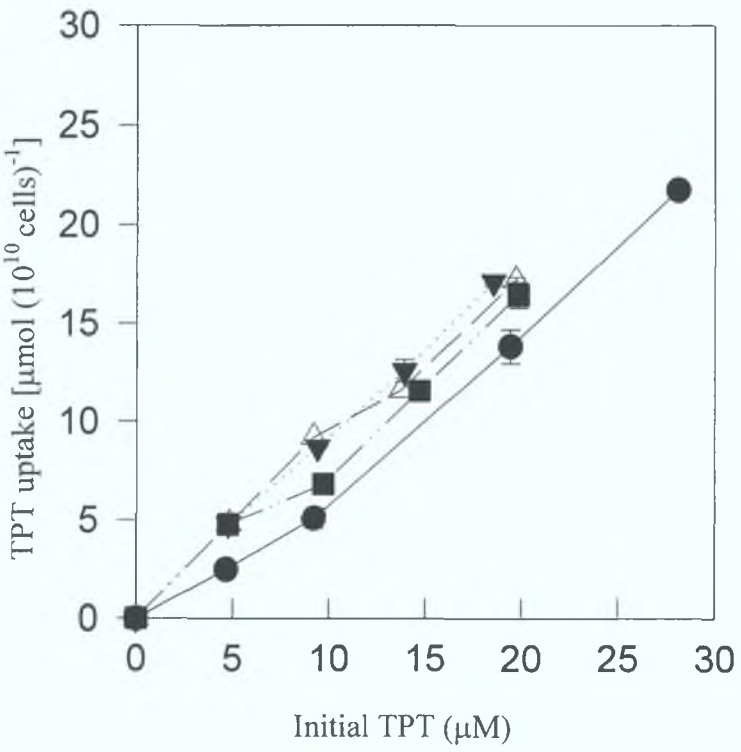

Figure 3.6.4 TPT uptake at pH 5.5 in 0 (●), 50 (■), 250 (▼) and 500 (Δ) mM NaCl. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

Loss in cell viability was enhanced considerably in NaCl (Fig. 3.6.5a and 3.6.6a). For TBT, complete cell death occurred at concentrations of 70, 50, 35 and 20 μM TBT in 0, 10, 50 and 500 mM NaCl, respectively. Exposure to 20 μM TPT resulted in 69, 39, and 6% cell viability in 0, 50 and 250 mM NaCl, respectively. In 500 mM NaCl, complete cell death resulted at concentrations greater than 10 μM TPT.

As mentioned in Section 3.6.2.1, the effects of NaCl itself on K^+ leakage cannot be discounted. These complicate K^+ results, as initial release from cells in 250 and 500 mM NaCl was consistently higher than at lower salinity, so no direct comparison between final release levels could be made. Also, representing values as the difference in K^+ release before and after exposure proved inaccurate as organotin-induced K^+ leakage did not follow a linear profile. Under certain conditions, K^+ release reached a level of 230 - 260 μmol $(10^{10}$ cells$)^{-1}$, which appeared to be the maximum possible level of K^+ leakage. Therefore, expressing K^+ release as the difference between initial and final concentrations would underestimate K^+ leakage under these conditions. However, taking this into account, an increase in organotin-induced K^+ leakage with increasing NaCl concentration was evident. For TBT, maximum K^+ release in the absence of NaCl occurred at 100 μM, while a similar level occurred at 50 and 20 μM TBT in the presence of 50 and 500 mM NaCl (Fig. 3.6.5b). Increased K^+ leakage was apparent even at low NaCl concentration, with higher K^+ levels in 10 mM NaCl compared to the absence of NaCl at all TBT concentrations. TPT-induced K^+ leakage also increased in the presence of NaCl (Fig. 3.6.6b). After exposure to 20 μM TPT, K^+ release levels reached 120, 200, 246 and 249 μmol $(10^{10}$ cells$)^{-1}$ in 0, 50, 250 and 500 mM NaCl.

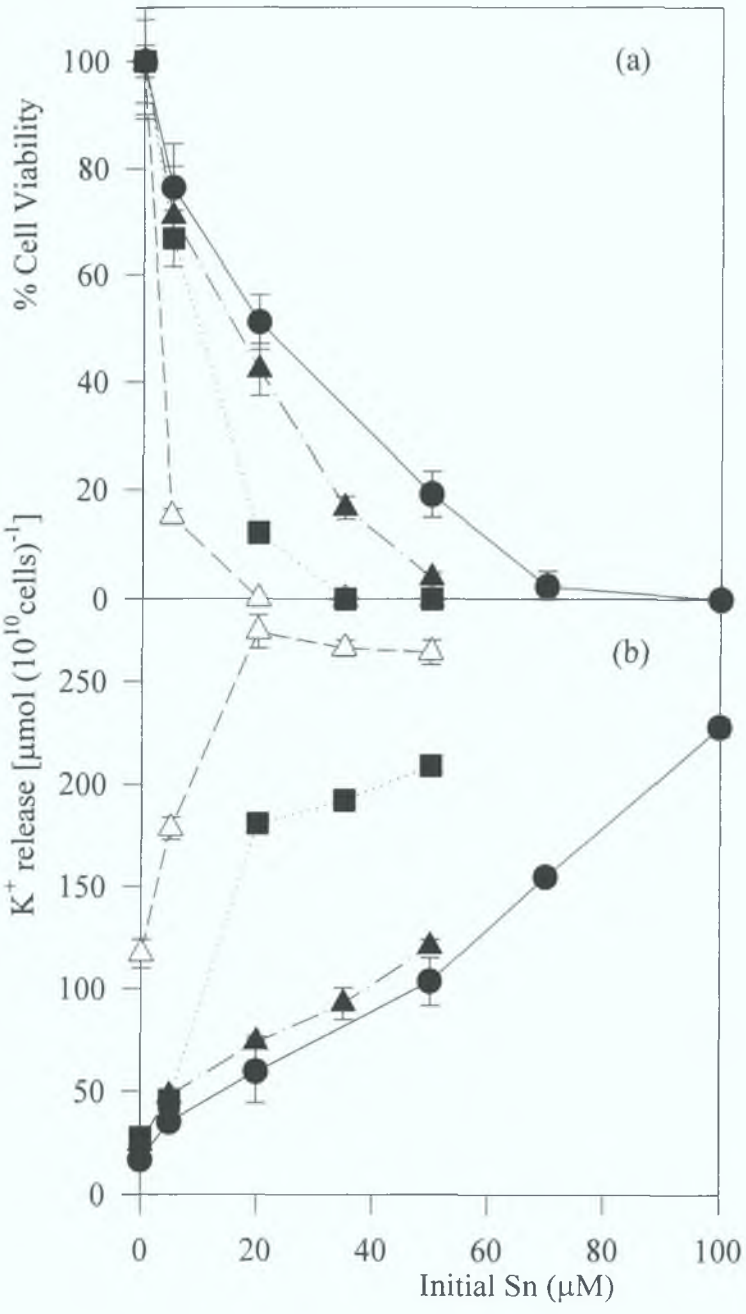

Figure 3.6.5 Influence of NaCl on the toxicity of TBT at pH 5.5. Cells were exposed to TBT in 0 (●), 10 (▲), 50 (■) and 500 (Δ) mM NaCl solutions and the resulting (a) loss in cell viability and (b) K^+ release was recorded. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

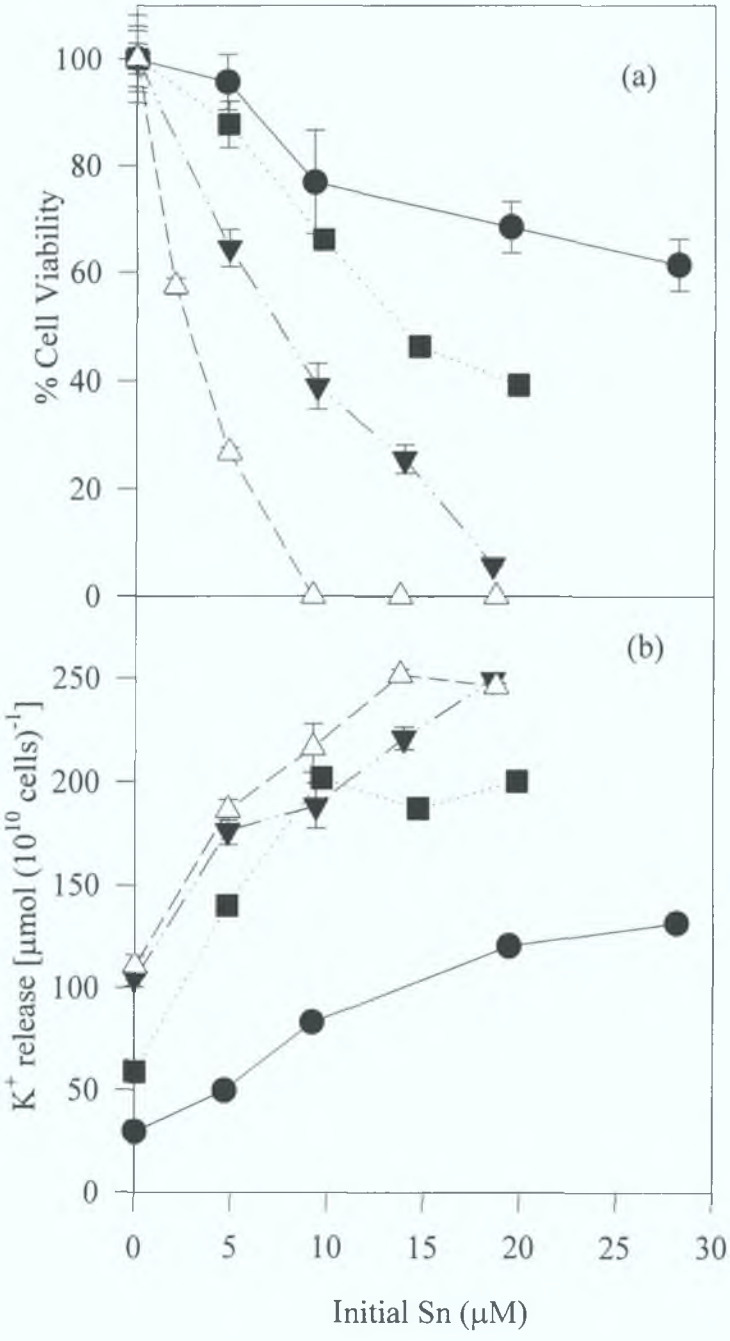

Figure 3.6.6 Influence of NaCl on the toxicity of TPT at pH 5.5. Cells were exposed to TPT in 0 (●), 50 (■), 250 (▼) and 500 (Δ) mM NaCl solutions and the resulting (a) loss in cell viability and (b) K^+ release was recorded. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

3.6.2.3 Influence of NaCl on the effects of TBT and TPT on membrane fluidity

The effects of TBT and TPT on DPH anisotropy were reduced in the presence of NaCl (Fig. 3.6.7 and 3.6.8). This was more apparent for TPT, as overall, it had a greater effect on DPH anisotropy. DPH anisotropy increased by 0.042 units after exposure to 20 μM TPT in the absence of NaCl. In comparison, the change in DPH anisotropy was reduced to 0.033, 0.019 and 0.017 in 50, 250 and 500 mM NaCl. At 50 μM TBT, the highest concentration examined in NaCl, a change in DPH anisotropy of 0.010 units was recorded in the absence of NaCl. This corresponded to changes of 0.009, 0.006 and -0.002 units in 10, 50 and 500 mM NaCl, respectively. In all cases there was no significant difference in TMA-DPH anisotropy.

There was no direct relationship between organotin-induced K^+ release and changes in membrane fluidity. TBT- and TPT-induced K^+ leakage increased with NaCl concentration. In contrast, changes in DPH anisotropy were reduced with increasing salinity, while TMA-DPH was unaffected.

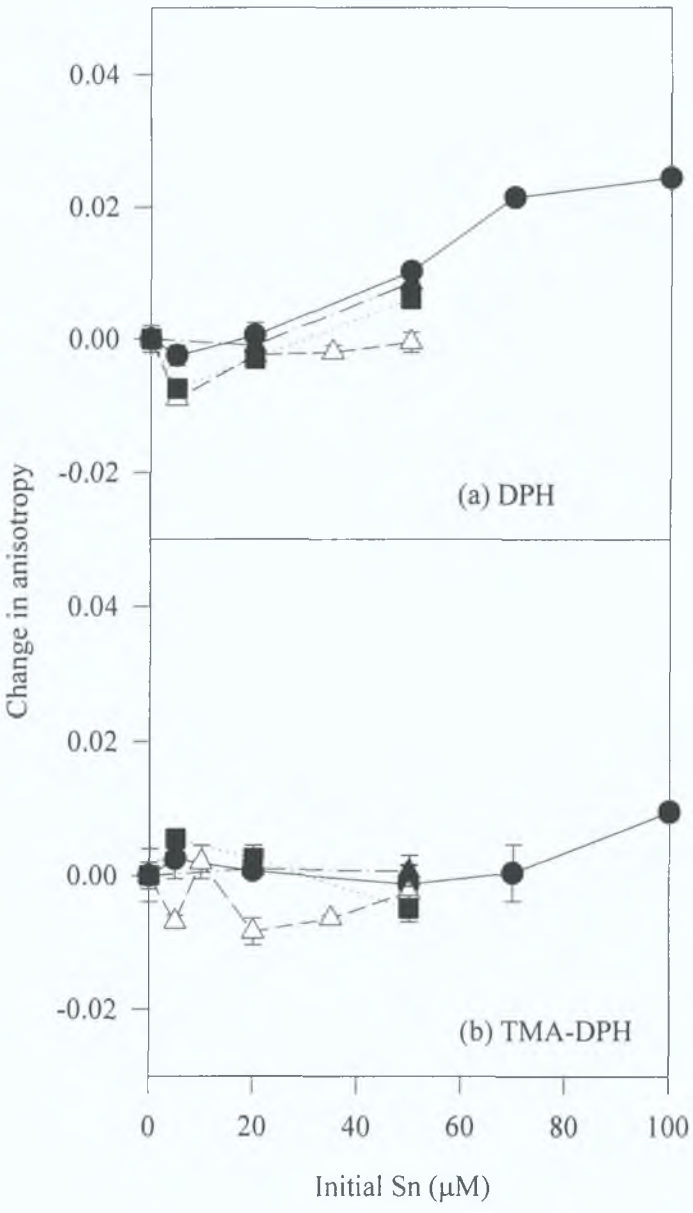

Figure 3.6.7 Influence of NaCl on the effect of TBT on the anisotropy of (a) DPH and (b) TMA-DPH. Cells were exposed to TBT in 0 (●), 10 (▲), 50 (■) and 500 (Δ) mM NaCl solutions and the resulting change in anisotropy was recorded. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

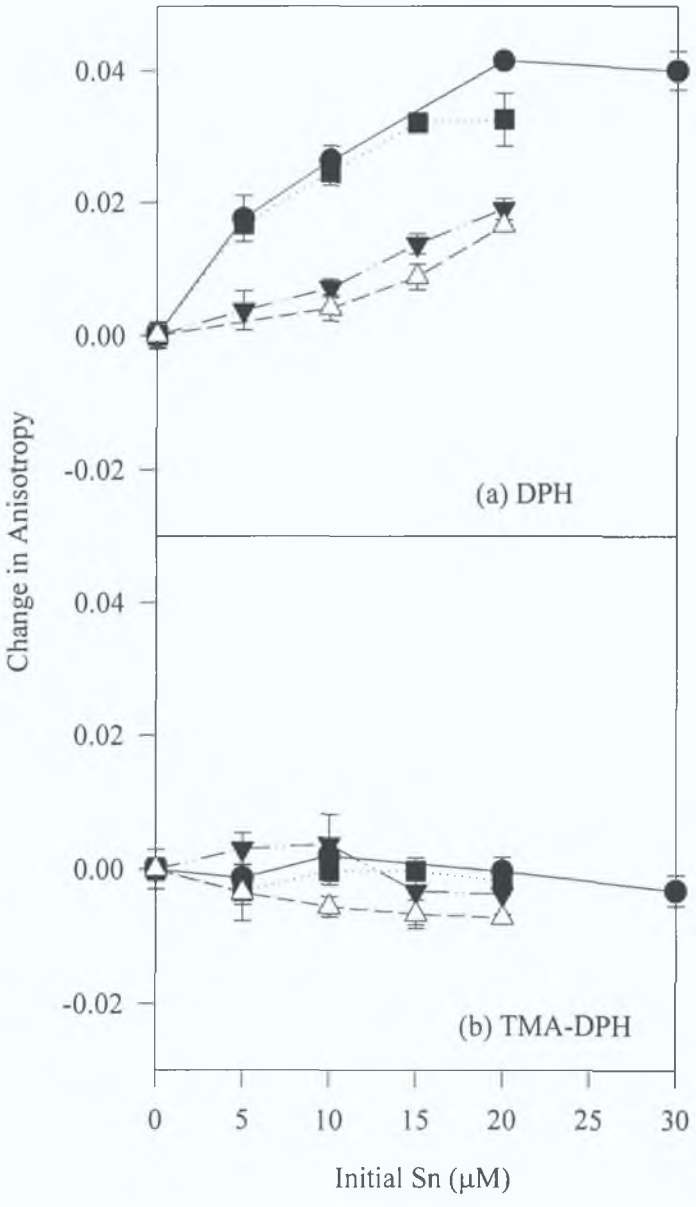

Figure 3.6.8 Influence of NaCl on the effect of TPT on the anisotropy of (a) DPH and (b) TMA-DPH. Cells were exposed to TPT in 0 (●), 50 (■), 250 (▼) and 500 (Δ) mM NaCl solutions and the resulting change in anisotropy was recorded. Mean values from three replicate determinations are shown ± SEM where they exceed the dimensions of the symbols.

3.6.3 Relationship between organotin toxicity, NaCl and D_{ow}

The relationship between organotin toxicity and NaCl concentration was examined. Cytotoxicity, as represented by 50 and 20% inhibitory concentrations of TBT and TPT, respectively, was compared to chloride concentration and D_{ow}. Inhibitory concentrations were calculated from Fig. 3.6.5a and 3.6.6a. A linear relationship was found between $\log_{10}$ [Cl^-] and IC values, with linear regression coefficients of 0.993 and 1.000, calculated for TBT and TPT, respectively (Fig. 3.6.9a). Cell toxicity was also related to lipophilicity, with a linear correlation between TBT and TPT inhibitory concentrations and D_{ow} values (Fig. 3.6.9b). Regression coefficients of 0.956 for TBT and 0.988 for TPT were calculated.

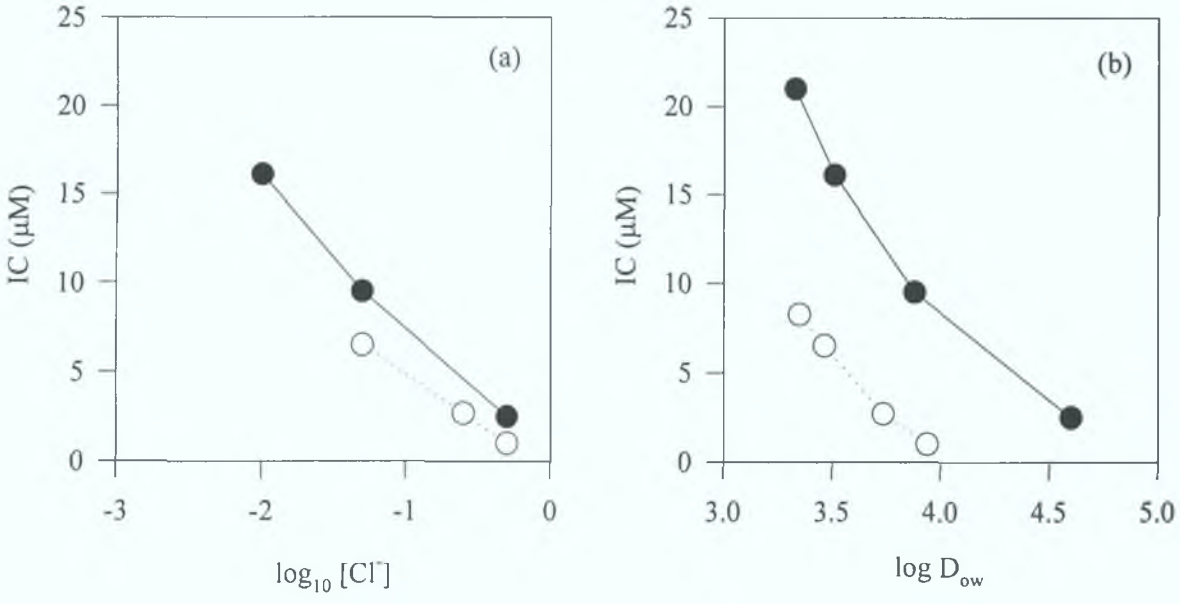

Figure 3.6.9 Variation in organotin cytotoxicity with (a) Cl^- (M) and (b) D_{ow}. TBT IC_{50} values (closed symbols) and TPT IC_{20} values (closed symbols) were determined from Fig. 3.6.5a and 3.6.6a, respectively.

3.7 Relationship between organotin toxicity and lipophilicity

The overall relationship between organotin toxicity and lipophilicity, as determined by the species present in solution, was investigated further. Firstly, the variation in inhibitory concentrations with D_{ow}, as predicted under different pH and NaCl concentrations were analysed (Fig 3.7.1). IC_{50} was used for TBT (from Fig 3.5.8b and 3.6.9b) while IC_{20} was used for TPT (from Fig 3.5.9b and 3.6.9b). The reason for this was that TPT was less soluble and for the majority of experimental conditions, 50% inhibition of cell viability was not reached. For TBT, IC_{20} was not suitable for determination of toxicity with varying NaCl concentrations as the concentrations of TBT resulting in 20% inhibition could not accurately be determined from Fig. 3.6.5. Hence, in order for all the experimental data for each compound to be compared different IC points had to be selected. There was a clear correlation between D_{ow} and cytotoxicity with regression coefficients of 0.860 and 0.975 for TBT and TPT, respectively. This also serves to underline the importance of speciation in assessing organotin toxicity. D_{ow} is an indication of how lipophilic an aqueous solution is, but it also reflects the fraction of neutral species present in solution.

Finally, all results were combined to investigate whether the relationship was specific for each compound or if there was a direct correlation between toxicity of both compounds and D_{ow} (Fig 3.7.2). Where possible, IC_{20} and IC_{50} values were determined for both compounds from Fig 3.5.4, 3.5.5, 3.6.5 and 3.6.6. Both IC_{20} and IC_{50} were reduced with increasing with D_{ow} values with linear regression coefficients of 0.803 and 0.852 respectively. These results indicate that a direct correlation exists between toxicity and overall lipophilicity, regardless of which compound was present in solution.

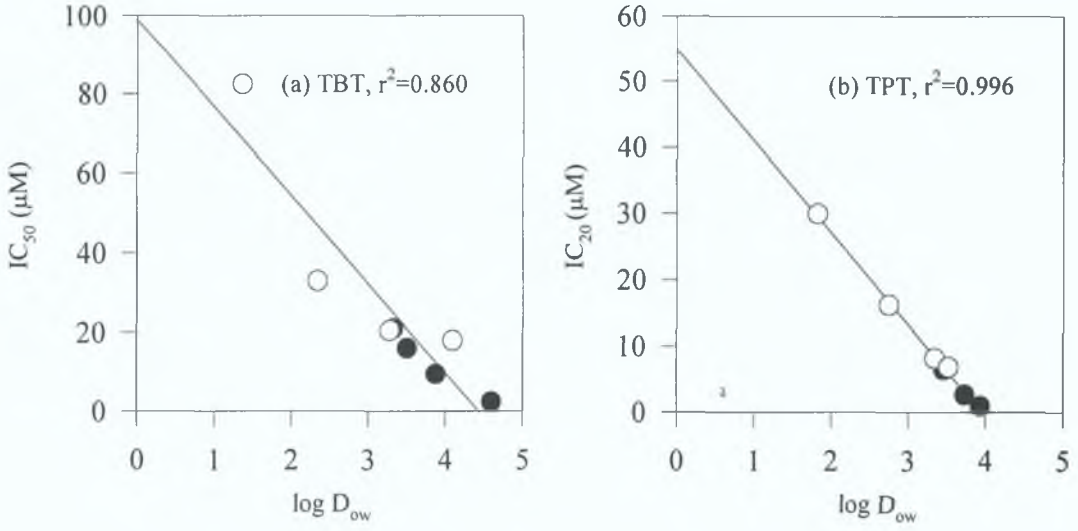

Figure 3.7.1 Relationship between (a) TBT and (b) TPT toxicity and D_{ow} values. TBT toxicity was determined as IC_{50} values and TPT toxicity as IC_{20} values. Open and closed symbols represent data calculated from pH and NaCl results, respectively. The D_{ow} values were determined at the corresponding experimental salinity and pH values. IC_{50} and IC_{20} correspond to the concentration of organotin causing 50 and 20% inhibition in cell viability, respectively.

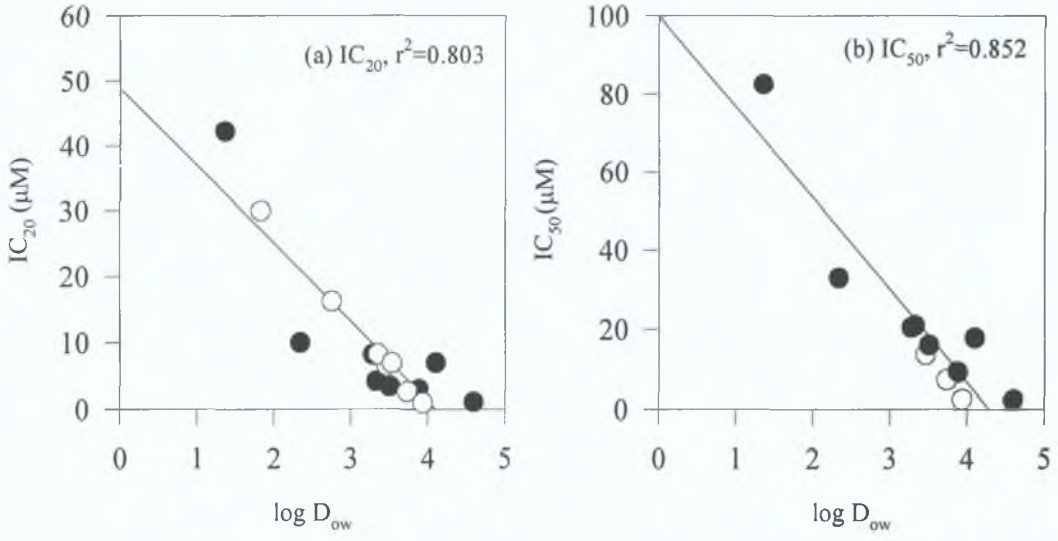

Figure 3.7.2 Overall correlation between organotin toxicity and D_{ow}. IC_{20} values were calculated from all TBT and TPT data, while IC_{50} values were determined from all TBT data and NaCl data for TPT. Open and closed symbols represent those points calculated from TPT and TBT results, respectively.

CHAPTER 4

DISCUSSION

Chapter 4: Discussion

4.1 Tin analysis

4.1.1 *Set-up of tin detection systems*

The most sensitive methods for analysis of organotins involve the conversion to alkyl derivatives or volatile hydrides and determination with specific detectors (Fent 1996b). For analysis of Sn(IV), TBT and TMT, a system using hydride generation followed by detection using AAS was developed based on that described by Dunne (1994) for the determination of arsenic compounds. Flow injection HGAAS was used with a 0.5 ml sample loop. The system had the advantages of being inexpensive with an analysis time of approximately 1 min for each sample and small sample volumes (~2 ml) were required. Using this system under optimum conditions (1 M HCl at a flowrate of 4.8 ml min^{-1}; 1% $NaBH_4$ at 4.0 ml min^{-1}; wavelength of 286.3 nm and air and acetylene settings of 5 and 2, respectively) detection limits of less than 0.4 μM were achieved. The detection limit considers both the signal amplitude and the baseline noise and is the lowest concentration that can be clearly differentiated from zero. Lower detection limits may be obtained by using larger injection volumes or altering the carrier gas flowrate. However, detection limits were adequate for the experiments described here.

TPT can not be detected using HGAAS, as conversion to hydrides is poor. Hence, TPT was analysed using differential pulse polarography. Organotin analysis using this method has been documented (Gadd et al., 1990; Avery et al., 1993) and TPT detection was sensitive enough for the purpose of these experiments. However, detection of TBT using differential pulse polarography was less sensitive, necessitating the use of HGAAS. Compared to polarography, smaller sample volumes were required for HGAAS (minimum of 2 ml compared to 30 ml) and the analysis procedure was more rapid and less tedious. Also, greater sample dilution was required for polarographic analysis to obtain the correct concentration of supporting electrolyte.

4.1.2 *TBT and TPT solubility*

In the absence of other ions, organotins exist as either R_3Sn^+ or R_3SnOH species, which have different solubility levels in solution. The solubility of TBT and TPT in distilled water varies with pH and shows a minimum in the pH range 6-8 (Inaba et al., 1995). To ensure that experiments were designed so that only soluble concentrations were examined, TBT and TPT solubility was determined at pH 5.5 and pH 7.5 (Table 3.1.4). Accordingly, the highest organotin concentrations examined were 100 μM TBT between pH 3.5 and 7.5, 30 μM TPT up to pH 5.5 and 10 μM TPT at pH 7.5. Overall, solubility values were higher than those previously reported (Inaba et al., 1995). The reason for this was the inclusion of methanol (up to 4%) as a co-solvent, as TBT and TPT were added from methanol stock solutions. The contribution of other solvents can only be discounted if the final concentration is less than 0.1% (Schwarzenbach and Gschwend, 1993).

When considering saline environments, the effects of the dissolved inorganic salt(s) on the aqueous solubility of organic compounds has to be taken into account. For neutral, nonpolar compounds, ionic species predominantly found in natural waters (i.e. Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, HCO_3^-) decrease aqueous solubility (Schwarzenbach and Gschwend, 1993). If one salt compound predominates in a salt mixture, as for example NaCl in seawater, it is acceptable to use the properties of that compound in place of the whole mixture. Organotin solubility decreased in the presence of NaCl. At pH 5.5, in 500 mM NaCl, the solubility of TBT and TPT was reduced to 65 and 24 μM respectively. As a result, 50 μM TBT and 20 μM TPT were the highest concentrations examined.

Despite the low solubility levels calculated here and those previously determined (Inaba et al., 1995), most of the reports to date have examined higher organotin concentrations, which may be insoluble under the specified experimental conditions (Cooney et al., 1989; Laurence et al., 1989; Avery et al., 1993). Hence, in these reports, the effect of pH and NaCl on organotin solubility can not be discounted when considering effects on organotin-microorganism interactions.

4.2 Localisation of DPH and TMA-DPH in intact cells and protoplasts

The time-dependent incorporation of TMA-DPH and DPH in *C. maltosa* was monitored by recording fluorescent anisotropy and intensity of the probes over a 70 min time period (Fig 3.2.1 and 3.2.2). DPH anisotropy reached steady-state after 30 min in intact cells and 10 min in protoplasts. TMA-DPH was rapidly localised in both cell systems. Anisotropy readings were stable between 30 and 70 min. Average readings of 0.133 and 0.096 for DPH and 0.269 and 0.229 for TMA-DPH were determined for intact cells and protoplasts, respectively. This confirmed that the probes were restrictively localised in cells during the time course of exposure to tin compounds in all experiments.

The membrane order of *C. maltosa*, expressed as the order parameter, S, which reflects the orderliness of membrane phospholipids, was calculated. S values of 0.825 and 0.761 for intact cells and protoplasts, was determined using TMA-DPH. The value for intact cells is similar to the range reported for *Schizosaccharomyces pombe* (S = 0.87 – 0.90) and *Saccharomyces cerevisiae* (S = 0.83 – 0.87) using the same probe (Gille et al., 1993). The membrane order at the location of DPH was less, at 0.606 and 0.515 for intact cells and protoplasts, respectively confirming that DPH was located at a more fluid region within the membrane. Overall, anisotropy of both TMA-DPH and DPH in protoplasts was lower than that in intact cells, indicating that the membrane fluidity of protoplasts was greater.

DPH and TMA-DPH are localised at different regions in cellular membranes. DPH is distributed between the hydrophobic region of lipidic membranes (Kuhry et al., 1983) and anisotropy reflects the average fluidity of all cellular membrane lipids (Swan and Watson, 1997). The charged derivative of DPH, TMA-DPH remains anchored at the head region of the phospholipid bilayer (Kuhry et al., 1983). In both intact cells and protoplasts, TMA-DPH anisotropy reflects the fluidity at the surface of the cytoplasmic membrane. As the fluorescent probes are localised at different sites, they are suitable for detecting interactions at different levels within the cell.

4.3 Comparison of interactions of TBT, TMT and Sn(IV) with *C. maltosa* at pH 5.5

The uptake of TBT, Sn(IV) and TMT by *C. maltosa* and subsequent toxic effects were compared at pH 5.5. TBT and Sn(IV) were removed from solution and interacted with *C. maltosa* by different mechanisms while, TMT did not become cell associated and no interactions were apparent. In intact cells, uptake of TBT by *C. maltosa* was twice that of Sn(IV). Moreover, uptake of inorganic tin had no effect on either cell viability or K^+ release, while TBT accumulation resulted in toxicity at all concentrations examined. For example, uptake of Sn(IV) to 40 μmol $(10^{10}$ cells$)^{-1}$ was not toxic while a similar TBT uptake level resulted in greater than 70% reduction in viability of both intact cells and protoplasts (Fig. 3.3.1 and 3.3.2).

Uptake of Sn(IV) was two-fold greater in intact cells than protoplasts (Fig. 3.3.1), indicating that cell wall binding was a dominant uptake mechanism. Maximum uptake levels were 40 and 19 μmol $(10^{10}$ cells$)^{-1}$ for intact cells and protoplasts, respectively. The maximum level of uptake observed for whole cells is consistent with the lowest uptake values reported in biosorption studies conducted over a range of generally higher tin concentrations (Tobin and Cooney, 1999). As Sn(IV) forms various cationic species in solution (Baes and Mesner, 1976) whole cell binding may be attributed to interactions with cell wall functional groups. In the absence of the cell wall, Sn(IV) may bind to charged groups on the surface of the cytoplasmic membrane, including carboxylate groups of membrane proteins. Less binding sites are available at the cytoplasmic membrane, leading to a reduction in uptake in protoplasts compared to intact cells.

K^+ release was unaffected by the presence and concentration of Sn(IV) in solution, suggesting that cytoplasmic membrane damage did not occur. The lack of cell death supports the view that cell integrity was not compromised. This is consistent with previous studies of the toxic effects of inorganic tins. At concentrations up to 0.8 mM, neither Sn(II) or Sn(IV) caused viability loss or K^+ leakage of *C. maltosa* at pH 2 or pH 3-4 (Tobin and Cooney, 1999). Similarly, inorganic tin was only toxic to the cyanobacteria, *Synechocystis aquatilis* under alkaline conditions (Pawlik-Skowrońska et al., 1997). At pH 9.0, 10 mg L^{-1} Sn(II) and Sn(IV) suppressed both the growth of the

cyanobacteria (by 54 and 26%, respectively) and the chlorophyll a content (by 58 and 24%, respectively), whereas, neither Sn(II) nor Sn(IV) exhibited any inhibiting effects at pH 7.0.

The finding that TBT uptake by whole cells and protoplasts, from initial TBT concentrations up to 100 μM, was essentially uniform was unexpected. Microbial biosorption of inorganic metals occurs primarily at the cell wall and groups such as carboxyl, phosphate and sulphydral are involved (Tobin et al., 1984). It would be anticipated that the removal of the cell wall would diminish TBT uptake in proportion to the contribution of cationic binding to negatively charged cell wall groups. However, TBT uptake was associated with extensive K^+ leakage and cell death indicating that membrane interactions resulting in structural damage were occurring. Similarly, TBT caused an increase in the fluorescence anisotropy of DPH in both intact cells and protoplasts, indicating a decrease in membrane fluidity. There was no discernible difference in TMA-DPH anisotropy. DPH anisotropy reflects the average fluidity of all cellular membrane lipids, while TMA-DPH only reports on the surface of the cytoplasmic membrane. Hence, TBT interactions were not confined to the surface of the membrane, but resulted in alterations in membrane fluidity within the phospholipid bilayer. Sn(IV) and TMT did not affect either probe, which confirms the absence of cytoplasmic membrane interactions.

Previous studies have focused on the interactions of TBT with model lipid membranes rather than whole cells. TBT increased the permeability of liposomes formed from egg phosphotidylcholine, causing efflux of dimethylarsinic acid (Cullen et al., 1997) while desorption of praseodymium ions from liposomes increased with TBT concentration (Gabrielska et al., 1997). The results presented here indicate that TBT uptake by yeast cells was not restricted to the cell wall and interactions within membrane lipids occurred. A similar site of action of TBT in liposomes has been suggested. Alteration in the thermotropic characteristics of dipalmitoyl phosphtidylcholine liposomes by TBT was more pronounced in the hydrophobic core region (Ambrosini et al., 1991a). Also, the action of TBT on the fluorescence polarisation of DPH and TMA-DPH in multilamellar liposomes was more marked in the core than in the head-group region (Ambrosini et al., 1991b).

Cell wall biosorption has previously been suggested as the main uptake mechanism of TBT by microorganisms (Blair et al., 1982; Gadd et al., 1990; Avery et al., 1993;). TBT uptake by *Aureobasidium pullulans* (Gadd et al., 1990), the cyanobacteria, *Synechocystis* PCC 6803 and *Plectonema boryanum* and the microalga, *Chlorella emersonii* (Avery et al, 1993) was attributed to adsorption to the cell surface as uptake was rapid and metabolism-dependent intracellular accumulation would have been inhibited by the high TBT concentrations. In these studies, cells were exposed to high organotin concentrations, between 0.5 and 1 mM. In the present work, organotin concentrations did not exceed 100 μM and diffusion mechanisms played a predominant role in TBT uptake as evidenced by similar uptake levels in protoplasts and intact cells. This indicated that cell wall biosorption is not necessary for uptake while the effects on membrane fluidity confirm the membrane-acting effects of TBT. In addition, as non-metabolising cells were examined over a short time period of 30 minutes, metabolic-dependent uptake can be discounted.

4.4 Influence of pH on TMT uptake and toxicity

TMT uptake, between pH 3.5 and 7.5, was negligible, while no effects on cell viability or membrane permeability were evident. The pKa values reported for TMT range from 5.79 to 6.60 (Arnold et al., 1997). Hence, both the TMT cationic (TMT^+) and neutral hydroxide species were considered to be present in the pH range investigated.

The lack of interaction between yeast cells and both species was unexpected. Biosorption of TMT^+ would be anticipated as TMT^+ forms complexes with different organic and inorganic cellular ligands such as amino acids, carboxylic acids and phosphate groups (Arnold et al., 1997). However, these results are consistent with previous work in which TMT, between pH 4.7 and 5.4, at concentrations up to 0.8 mM, did not interact with *C. maltosa* (Tobin and Cooney, 1999). Also, uptake of TMT, from an initial concentration of 0.5 mM at pH 5.5, by the cyanobacteria *Plectonema boryanum* was negligible (Avery et al., 1993). TMT was also the least toxic organotin towards *E. coli* with LC_{50} values (concentration at which 50% reduction in growth occurred) of 337, 1.32 and 2.86 μM for TMT, TBT and TPT, respectively (Eng et al., 1991). At higher pH, lipophilic interactions between neutral TMT species and cells would also be expected. However, TMT has the least effect on planar lipid bilayers, with organotins of increased alkyl chain length resulting in a higher degree of membrane depolarisation (Radecka et al., 1999). Similarly, methyltins have little or no effect on membrane permeability of *Debaryomyces hansenii* (Laurence et al., 1989).

4.5 Influence of pH on speciation and interactions of TBT and TPT with *C. maltosa*

Knowledge of the aqueous speciation of organotins is essential in assessing bioavailability and toxicity of these compounds. Organotins may undergo pH-dependent hydrolysis when introduced into water. Cations are dominant at pH less than pK_a and these monovalent organometallic cations behave as weak acids, while at pH above pK_a, neutral species dominate. The variation in TBT and TPT speciation with pH (Fig. 3.5.1) was predicted using data from Arnold et al. (1997). R_3SnOH species were assumed to be the only neutral species present in solution as all other monovalent anions form relatively weak complexes with both TBT and TPT (Arnold et al., 1997). For example, formation constants (log K_i) for Cl^- (organotins were added as chloride salts) and NO_3^- (HNO_3 was used for pH adjustment of buffers) are 0.60 and 0.62 for TBT and 0.66 and 0.26 for TPT (Arnold et al., 1997). In contrast the log K_i values for TBTOH and TPTOH are 7.75 and 8.80, respectively.

The D_{ow} values of organotins also vary with pH (Fig 3.5.2). At pH 3.5, where few neutral species are present, the D_{ow} of TBT and TPT were 1.36 and 0.82, respectively. These increased to a maximum of 4.1 at pH 7 for TBT and 3.53 at pH 6 for TPT and remained constant above these pH values. The D_{ow} values that were predicted below pH 5.5 are slightly lower than those determined experimentally by Arnold et al. (1997). This is likely due to their experimental aqueous solution containing 10 mM $NaClO_4$, which would contribute to formation of neutral species at low pH and also alter the ionic strength used in calculations.

The variation in organotin uptake and toxicity with pH and the relationship with compound lipophilicity was examined. The contribution of the individual R_3Sn^+ and R_3SnOH species as influenced by pH was also assessed. Organotin uptake and effects on viability and membrane integrity varied considerably with external pH. TBT uptake levels were similar between pH 4.5-7.5, but were reduced at pH 3.5, while uptake of TPT increased from pH 3.5 to 5.5. Organotin-induced cell death and K^+ release increased with pH. Increased toxicity was not necessarily due to higher uptake levels, but was also dependent on the organotin species present in solution. Changes in external pH can influence the uptake and toxicity of metals in at least two ways: (i) by affecting

metal speciation and (ii) by its effect on the cell surface. The contribution of these two factors was assessed at each pH.

Uptake and toxicity of TBT and TPT were reduced considerably at pH 3.5. At this pH, greater than 98% R_3Sn^+ species are present and D_{ow} values are low. Consequently, uptake mechanisms similar to biosorption of cationic metals would be expected. Uptake of cationic metals in non-metabolising systems is generally attributed to ion exchange, adsorption, complexation, precipitation and crystallisation to the cell surface (Tobin et al., 1984; Gadd 1993). The adsorptive capacity of the yeast cell for metals is determined by the degree of dissociation of the negatively charged functional groups. As the surface of the cell alters with pH, the effect of this on uptake has to be taken into account. A pH between 4.0 and 8.0 is widely accepted as being optimal for metal uptake, while below this pH, protonation of possible binding sites becomes significant and adsorption is reduced (Blackwell et al., 1995). Uptake of both TBT and TPT was reduced considerably at pH 3.5, compared to 4.5, implying that competition with H^+ ions was significant and resulted in a reduction in uptake.

TBT interactions differed considerably between pH 3.5 and 4.5. TBT uptake levels and toxicity, as indicated by loss in cell viability and K^+ release were higher at pH 4.5. One reason for this is the effect of pH on the cell surface resulting in greater uptake at higher pH, as discussed above. However, the difference in toxicity is not due solely to increased uptake. Cell death and K^+ release differed even at similar uptake levels. Maximum uptake at pH 3.5 of 66 μmol $(10^{10}$ cells$)^{-1}$ from an initial concentration of 100 μM TBT resulted in 27% cell viability and in a difference in K^+ release, before and after exposure, of 15 μmol $(10^{10}$ cells$)^{-1}$ (Fig. 3.5.4). In contrast, uptake of 70 μmol TBT $(10^{10}$ cells$)^{-1}$ at pH 4.5 resulted in 16% cell viability and a change in K^+ leakage of 103 μmol $(10^{10}$ cells$)^{-1}$. Also, TBT had no effect on fluorescent anisotropy at pH 3.5, while TMA-DPH anisotropy decreased at pH 4.5 (Fig. 3.5.6). As TMA-DPH is located at the surface of the cytoplasmic membrane, this indicated that TBT uptake disrupted membrane integrity at this region at pH 4.5. The lipophilicity of TBT increased with pH, with D_{ow} values of 1.36 and 2.34 at pH 3.5 and 4.5, respectively. Thus, cytoplasmic membrane binding sites were more readily accessed at the higher pH, resulting in increased membrane fluidity and cell toxicity.

TPT uptake was less than TBT at pH 4.5. Maximum uptake of TPT was 15 μmol $(10^{10}$ cells$)^{-1}$ from a 30 μM solution, while most of TBT was removed at all initial concentrations. However, effects on cell toxicity were similar at equivalent uptake levels. Comparisons between membrane fluidity effects could not be made due to the low concentrations of TPT examined. Effects of TBT on fluorescence anisotropy were only apparent after uptake of 20 μmol $(10^{10}$ cells$)^{-1}$, while maximum uptake of TPT was 15 μmol $(10^{10}$ cells$)^{-1}$.

Organotin toxicity increased at pH 5.5. For TPT, viability was reduced to 81, 70 and 62% after exposure to 30 μM TPT, at pH 3.5, 4.5 and 5.5, respectively (Fig. 3.5.5). Membrane interactions were greater, with extensive K^+ release at pH 5.5 compared to minimal changes in external K^+ concentrations at lower pH. DPH anisotropy also increased with TPT concentration at pH 5.5, indicating a reduction in fluidity at the hydrophobic core of membrane lipids (Fig. 3.5.7). The change in TPT toxicity corresponded to the increase in neutral hydroxide species present in solution. Between pH 4.5 and 5.5, the neutral fraction of TPT increased from 16 to 66%, while D_{ow} increased from 2.75 to 3.35. Consequently, greater membrane interactions and subsequent cell death were associated with the increased level of lipophilic species present in solution.

A similar variation in TPT toxicity occurred at pH 7.5, where 99.5% of TPT was present as TPTOH and a D_{ow} of 3.53 was predicted. However, comparison with other pH values was only possible at low organotin concentrations due to the reduction in solubility. At pH 7.5, 10 μM TPT resulted in a slight increase in cell death and K^+ release compared to pH 5.5 while there were similar changes in DPH anisotropy.

Toxicity of TBT was greater at pH 5.5 than 4.5 despite the similar uptake levels. TBT also had a very different effect on membrane fluidity at the higher pH. At pH 5.5, the anisotropy of DPH increased at concentrations above 20 μM while TMA-DPH anisotropy was unaltered. This indicated that at pH 5.5, TBT disturbed the hydrophobic core of membranes, while there was no interaction with the surface of the cytoplasmic membrane. At pH 5.5, 15% of TBT was present as neutral TBTOH, with a D_{ow} value of 3.28 compared to 2% TBTOH and a D_{ow} of 2.24 at pH 4.5. The higher lipophilicity and presence of greater neutral species would enable greater membrane interactions.

The increase in TBT toxicity at pH 7.5 compared to pH 5.5 was less than that which would be predicted by aqueous speciation considerations. At pH 5.5, 15% TBTOH was present, increasing to 95% at pH 7.5, while the D_{ow} of TBT increased from 3.28 to 4.1. If TBTOH were more biologically active that TBT^+, a substantial increase in cell death would be expected. There was a slight change in cell viability, with complete cell death occurring at 70 and 50 μM TBT at pH 5.5 and 7.5, respectively. However, membrane interactions were actually reduced at pH 7.5. Above 50 μM TBT, K^+ leakage was less at the higher pH, while there was no effect on membrane fluidity. These findings suggest that although similar toxicity resulted, the combination of TBT^+ and TBTOH species present at pH 5.5 interacted differently with cells compared to TBTOH at pH 7.5.

To summarise the effect of pH on organotin toxicity, the variations in IC_{50} and IC_{20} for TBT and TPT, respectively, were calculated. Two inhibition percentages were chosen as 50% cell death did not occur after exposure to TPT at the concentrations allowed by its low solubility. The increase in TPT toxicity with pH was clearly apparent, with over a four-fold decrease in IC_{20} between pH 3.5 and 7.5 (Fig. 3.5.9). The relationship between toxicity and lipophilicity, as indicated by D_{ow} was also investigated. There was a linear correlation between D_{ow} and IC_{20}. This clearly demonstrated that TPT toxicity was dependent on speciation, with TPTOH being more toxic than TPT^+. The relationship between TBT toxicity and pH was not as simple as that of TPT. IC_{50} did decrease with pH but toxicity at pH 5.5 was similar to pH 7.5 with IC_{50} of 20 and 18 μM, respectively (Fig. 3.5.8). This was unexpected as the fraction of neutral species increased from 15 to 95% between pH 5.5 and 7.5. Consequently, the correlation between D_{ow} and IC_{50} was also non-linear. Furthermore, these results underline the limitations in using partitioning models as toxicity indicators. The 1-octanol-water system is suitable for assessing the partitioning behaviour of neutral organic compounds between natural organic phases and water (Schwarzenbach and Gschwend, 1993) and has been used to estimate bioconcentration in organisms (Mackay, 1982). However, in the present system charged species are not accounted for in the partitioning calculations. Biological systems exhibit both distinct hydrophobic and hydrophilic regions where ionic and neutral species of a compound may interact by

different mechanisms. Octanol-water partition coefficients exclude the contribution of charged species, so in this case the toxicity of the combination of TBT^+ and TBTOH at pH 5.5 would be underestimated.

There is limited research on the influence of pH on the biological activity of organotins. Results are conflicting and comparisons are hampered by the different experimental systems employed (Table 4.5.1). Many studies use microorganisms exposed to organotins in the presence of glucose so metabolic-dependent interactions can not be discounted, while exposure to organotins in complex media does not take the influence of possible ligands into account. However, TBT uptake by microorganisms has been shown to vary with pH (Avery et al., 1993). Maximum uptake of 500 μM TBT by the cyanobacteria, *Synechocystis* PCC 6803 and *P. boryanum* occurred between pH 5.5-6.5. Reduced uptake below pH 5.5 was attributed to the competition with H^+ for binding sites, while the TBT hydroxide species was suggested to have decreased biological activity, resulting in less uptake above pH 6.5. This is in direct contrast with the results presented here, where TBT uptake levels were similar above pH 4.5, albeit at lower exposure concentrations. Insolubility of TBT at the higher pH could account for the decreased uptake levels, as cells were exposed to 500 μM TBT (Avery et al., 1993). TBT has a solubility of 134 μM in distilled water at pH 7.9, when added as a pure solution (Inaba et al., 1995) and the solubility level did not exceed 150 μM at pH 7.5 when added from a methanol stock solution (Table 3.1.4).

Table 4.5.1 Summary of the effect of pH on organotin interactions.

Experimental system	Effect observed	Ref.
Effect of TBT on growth of *A. pullulans* (between pH 5.5-7) and *R. rubra* (between pH 7.2-8.4) on two-dimensional gradient plates.	*A. pullulans*: TBT toxicity decreased from pH 5.5 to 7.0. *R. rubra*: TBT least toxic at pH 7.7-7.9.	Cooney et al., 1989
Exposure of *Debaromyces hansenii* to 50 μM organotin in pH 4-9 buffers containing glucose.	Maximum K^+ release at pH 6.5 for TBT, MBT and TPT and at pH 5.0 for DBT.	Laurence et al., 1989.
Growth of *A. pullulans* in media containing 0.3 μM TBT.	TBT was more toxic at pH 4.0 compared to pH 5.2.	Gadd et al., 1990
Uptake of 1 mM TBT by *A. pullulans* from liquid media, at between pH 2.5 to 6.5.	TBT uptake unaffected between pH 3.5-6.5, Maximum uptake was reduced by 20% at pH 2.5.	Gadd et al., 1990
Uptake of 0.5 mM TBT by the cyanobacteria, *Synechocystis* PCC6803 and *P. boryanum* and the microalga, *C. emersonii* after 30 min incubation in pH 4.5-8.5 buffered solutions.	Maximum uptake by *Synechocystis* and *P. boryanum* occurred at pH 5.5 and 6.5, respectively. No effect on uptake by *C. emersonii.*	Avery et al., 1993
Exposure of *Daphnia magna* to TBT at pH 6 and pH 8.	Bioconcentration and toxicity of TBT was significantly enhanced at pH 8 compared to pH 6.	Fent and Looser, 1995
Bioconcentration of TBT, TPT and tetrabutyltin (TeBT) in the sediment organism, *Chironomus riparius* at pH 5 and 8.	The bioconcentration factor (BCF) of both TBT and TPT was higher at pH 8 compared to pH 5. No difference for TeBT.	Looser et al., 1998

R_3SnOH species have been shown to be more biologically active than R_3Sn^+ species. TBT uptake rates, bioaccumulation and mortality are significantly higher in *Daphnia* at pH 8 than 6 (Fent and Looser, 1995). Similarly, increased bioconcentration of TBT and TPT in the sediment organism, *Chironomus riparius* at pH 8 compared to 5 is explained by the greater uptake of R_3SnOH species (Looser et al., 1998). Bioconcentration of TeBT, which does not dissociate in water, is not influenced by pH, confirming that the H^+ concentration has no significant influence at the lower pH. However, the difference in bioconcentration is less than that predicted by the octanol-water partition model. As discussed above, the K_{ow} model is often used to estimate the uptake of organic compounds (Schwarzenbach and Gschwend, 1993) but fails to take into account the interaction of charged species with biological membranes. Similarly, in the results presented here TBT uptake levels were independent of speciation and D_{ow} values between pH 4.5 and 7.5, underlining the limitations of using D_{ow} values for predicting interactions.

Organotin effects on membrane fluidity, as monitored by DPH and TMA-DPH anisotropy, varied with pH and speciation. Fluorescence anisotropy reports the rotational diffusion of the probes, which depends on the relative mobility of the phospholipid acyl chains in the immediate region. TPT had no effect at pH 3.5 or 4.5 but caused an increase in DPH anisotropy at pH 5.5 and 7.5. This implies that TPT^+ did not alter membrane fluidity while TPTOH caused an increase in order of the fatty acyl chains in the hydrophobic core of the bilayer. In comparison, localisation of TPT in the hydrophobic core of the phospholipid bilayer has been suggested, with TPT, at equivalent concentrations to those investigated here, causing an increase in DPH anisotropy in liposomes (Ambrosini et al., 1996). This is similar to the effect of organophosphorus insecticides (Blasiak, 1995; Antunes-Madeira et al., 1996). The membrane location of the insecticides facilitate hydrogen bonds or dipole-dipole interactions between the compounds and the fatty acyl chains resulting in a decrease in lipid spacing and hence, increased ordering of the membrane. This ordering of the fatty acyl chains would alter the physical state of the boundary lipid that surrounds membrane proteins and consequently protein conformation and function. However, adsorption of TPT to the membrane lipid/water interface with limited disturbance of the hydrophobic

interior has been suggested (Langner et al., 1998, 2000a and 2000b). In the results presented here, the absence of TPT interactions at the surface of the cytoplasmic membrane is evident with no changes in TMA-DPH anisotropy detected at exposure concentrations of up to 30 μM.

The action of TBT on membrane fluidity was more complicated. At pH 4.5, TBT caused a decrease in TMA-DPH anisotropy, reflecting an increase in membrane fluidity at the headgroup region. At this pH, greater than 98% of TBT is present as TBT^+ and an increase in fluidity is consistent with partial insertion of the alkyl chains of TBT in the phospholipid bilayer. Compounds that adsorb at the lipid-water interface, with partial penetration of the lipid bilayer cause a marked fluidisation effect (Różycka-Roszak et al., 2000). At pH 5.5, TBT caused an increase in DPH anisotropy, although to a lesser degree than TPT. The highest levels of TBT-induced K^+ release also occurred at this pH. In contrast, at pH 7.5, with greater than 95% of species as TBTOH, there were no membrane fluidity effects.

TBT and TPT hydroxide species interacted differently with cells. TPTOH caused a decrease in fluidity at the hydrophobic core, whereas the lack of TBTOH effects suggests that it does not accumulate in the cytoplasmic membrane. Diffusion of TBTOH across the cytoplasmic membrane may account for the absence of membrane fluidity effects and reduction in K^+ release. Dissolution of lipophilic organic metal complexes in the membrane, followed by diffusion across the lipid bilayer and distribution among cellular compartments has been demonstrated (Phinney and Bruland, 1994). The steric constraints of the phenyl groups of TPTOH may prevent diffusion of TPT through the membrane (Langner et al., 2000a).

4.6 Influence of NaCl on speciation and interactions of TBT and TPT with *C. maltosa* at pH 5.5

Based on the pH-dependent speciation profiles presented for TBT and TPT, altered toxicity in differing aqueous environments would be expected. For example, greater bioaccumulation in marine species compared to freshwater species has been reputed (Meador, 2000). This may be due partly to the fact that the pH of freshwater is generally lower than that of marine waters so higher levels of R_3SnOH species in marine waters would lead to higher bioaccumulation. However, this analysis does not take the presence of other ions into consideration. In this section, the influence of NaCl, the most dominant ion in marine systems, on organotin-yeast interactions was assessed.

In aqueous solution, triorganotins dissociate to form R_3Sn^+ and R_3SnOH species. In the presence of NaCl, R_3SnCl species may also form. Using the data provided by Arnold et al., (1997) the speciation of TBT and TPT under various ionic conditions at pH 5.5 was predicted. This pH was chosen so that a broad variation in speciation over the NaCl concentration range would result, allowing the contribution of the individual species to be assessed. At higher pH values, R_3SnOH is predominant and a higher concentration of NaCl would be required for formation of R_3SnCl, while a lower pH would favour formation of cationic species and the presence of H^+ ions would have to be taken into account. At pH 5.5, in the absence of NaCl, it was predicted that TBT would be composed of 85% TBT^+ and 15% TBTOH with TPT present as 33% TPT^+ and 67% TPTOH (Fig. 3.6.1). Increasing the NaCl concentration resulted in the formation of chloride species. In 500 mM NaCl, 62% TBT^+, 4% TBTOH and 34% TBTCl and 36% TPT^+, 35% TPTOH and 29% TPTCl was predicted.

The variation in organotin species also alters D_{ow} values (Fig. 3.6.2). Chloride species are more lipophilic than the corresponding hydroxide species with log K_{ow} of 4.76 and 4.10 for TBTCl and TBTOH and 4.19 and 3.53 for TPTCl and TPTOH, respectively (Arnold et al., 1997). Consequently the formation of neutral chloride species results in increased D_{ow} values. In the absence of NaCl at pH 5.5, the D_{ow} of TBT and TPT was calculated as 3.28 and 3.35, respectively, whereas, in 500 mM NaCl, values of 4.60 for TBT and 3.94 for TPT were determined.

The effect of NaCl on *C. maltosa* was assessed prior to investigation of the influence of NaCl on organotin-yeast interactions. After 30 min exposure, NaCl, at concentrations up to 500 mM, had negligible effect on cell viability and membrane fluidity, while K^+ release to a level of approximately 130 μmol $(10^{10}$ cells$)^{-1}$ was induced. This was attributed to the non-specific leakage of ions from the cytosol due to osmotic changes in the cell. Active transport of K^+ across the membrane could be discounted as experiments were performed with non-metabolising cells. Generally, NaCl is not toxic to yeast below 1 M (Aguiar and Lucas, 2000) so no loss in viability would be expected. Alteration in membrane composition of cells grown in the presence of NaCl, resulting in increased fluidisation has been observed (Hosono, 1992; Khaware et al., 1995). However, membrane adaptation mechanisms were not expected here as cells were exposed for only 30 min in the absence of a carbon source. The similarity between anisotropy values of DPH and TMA-DPH after exposure to 0, 50 and 500 mM NaCl confirmed the absence of membrane fluidity changes. These results indicate that in the following experiments, the influence of NaCl on organotin toxicity was due to the changes in organotin-yeast interactions and deleterious effects of NaCl on the yeast could be discounted. Only in the case of K^+ release had the action of NaCl to be accounted for, as K^+ leakage levels in the absence of organotin increased with NaCl concentration.

There was little difference in TBT uptake between 0 and 500 mM NaCl at pH 5.5 with almost complete removal of concentrations of up to 50 μM from solution. There was a marginal increase in TPT uptake between 0 and 50 mM NaCl, but there was no discernible difference at higher concentrations. Maximum uptake levels of 13.8, 16.5, 17.1 and 17.2 μmol $(10^{10}$ cells$)^{-1}$ were determined in 1, 50, 250 and 500 mM NaCl. This result was expected, as there would be very little competition between Na^+ and R_3Sn^+ for potential binding sites as organotin concentrations were well below those that would result in saturation of sites. Uptake of TBT and TPT by *C. maltosa* did not reach saturation from initial concentrations of up to 0.4 mM (Tobin and Cooney, 1999). NaCl would have little influence on uptake of neutral species, as different interaction mechanisms would be expected. In contrast, the only other study to consider the effect of NaCl on organotin uptake reported a reduction in TBT uptake levels in 500 mM NaCl

(Avery et al., 1993). Uptake of 500 μM TBT by *Synechocystis* PCC 6803, *P. boryanum* and *C. emersonii* at pH 5.5 was reduced by 55-65% in 500 mM NaCl (Avery et al., 1993). In this case reduced uptake may be due to the effect of Cl^- on solubility, as the initial organotin concentration was ten times the limit of solubility reported here. TBT was added from an ethanol stock solution, with final ethanol concentrations of 1-3%, so similar solubility levels would be expected.

Organotin-induced cell death was enhanced considerably in the presence of NaCl, even though uptake levels were similar (Fig. 3.6.5a and 3.6.6.a). As the effects of NaCl itself could be discounted, enhanced toxicity was associated with the combination of organotin species, as influenced by salinity. The change in toxicity with NaCl concentration may be due to either the overall increased fraction of neutral species as the less toxic cationic fraction is reduced or the difference in biological activity of the individual species. For TPT it was predicted that the fraction of neutral TPT species (TPTOH and TPTCl) remained relatively constant (67-63%) as TPTCl was formed in place of TPTOH. This indicates that it was the increasing fraction of chloride species that resulted in enhanced toxicity. For TBT, both the fractions of total neutral and chloride species increased with NaCl concentration, with 15 and 38% neutral species in 0 and 500 mM NaCl, respectively. However, TBT was more toxic in 500 mM NaCl, pH 5.5, where the fraction of TBTOH was reduced to 4% with 34% TBTCl, than at pH 7.5, where 95% TBTOH was present. Complete loss in cell viability occurred at 20 μM TBT in 500 mM NaCl, pH 5.5, compared to 50 μM at pH 7.5, which suggests that TBTCl was more toxic than TBTOH. Thus, for TBT and TPT, the fraction of species present in solution influenced cell toxicity with the neutral species being more toxic than the cationic species and chloride species being more toxic than the hydroxide species.

Organotin-induced K^+ release increased with NaCl concentration, irrespective of the higher NaCl control release levels. K^+ release levels reached approximately 104, 121, 209, 264 μmol K^+ $(10^{10}$ cells$)^{-1}$ after exposure to 50 μM TBT in 0, 10, 50 and 500 mM NaCl (Fig. 3.6.5). After exposure to 20 μM TPT in 0, 50, 250 and 500 mM NaCl, K^+ release levels reached 120, 200, 246 and 249 μmol $(10^{10}$ cells$)^{-1}$ (Fig. 3.6.6). K^+ release results were complicated by the higher control K^+ release levels in the presence of NaCl so no direct correlation between K^+ leakage effects and salinity could be made.

Even so, these results show that the action of organotins on membrane permeability was considerably enhanced in the presence of NaCl. In contrast, organotin-induced K^+ leakage from *D. hansenii* (Laurence et al., 1989) and *Z. rouxii* (Cooney et al., 1989) was reduced in the presence of 1.5 and 3% NaCl at pH 6.5. However, in those works the reduction in K^+ leakage may be attributed to the effect of NaCl on solubility of 50 μM organotin at pH 6.5, while the influence of metabolism-dependent interactions could not be discounted as cells were exposed to organotins in the presence of glucose.

Organotin effects on DPH anisotropy were reduced in the presence of NaCl. This was more apparent for TPT, as overall, it had a greater effect on DPH anisotropy. At pH 5.5, in the absence of NaCl, 20 μM TPT resulted in an increase in DPH anisotropy by 0.042 units, implicating the action of TPT at the hydrophobic core of membrane lipids. Under these conditions, it was predicted that TPT consisted of 33% TPT^+ and 67% TPTOH. This effect may be attributed to the action of TPTOH because TPT^+ did not alter membrane integrity, as evidenced by the absence of membrane fluidity changes at pH 3.5 and 4.5. As the NaCl concentration increased, corresponding to the formation of TPTCl in place of TPTOH, the effect of 20 μM TPT on DPH anisotropy was reduced, with changes of 0.033, 0.019 and 0.017 in 50, 250 and 500 mM NaCl (Fig. 3.6.8). This indicates that TPTCl and TPTOH have different effects on membrane lipids. TPTOH interacted with lipids at the hydrophobic core of the phospholipid bilayer, as evidenced by the greatest effect on DPH anisotropy in the absence of NaCl. In contrast, TPTCl did not affect DPH anisotropy, as indicated by a reduction of the overall effect with increasing Cl^- concentration. A similar interaction was observed for TBT, although this was not as apparent as overall TBT had less effect on DPH anisotropy at pH 5.5 (Fig. 3.6.7). However, the absence of TBTCl effects on membrane fluidity was clearly obvious.

The difference in organotin effects on K^+ release and membrane fluidity, as reported by changes in DPH and TMA-DPH anisotropy suggests that there is no exact relationship between these two parameters in monitoring membrane interactions. Anisotropy of fluorescent probes only relates to the fluidity of the immediate region where they are located. DPH intercalates between the lipids in the hydrophobic tail region of the phospholipid bilayer (Shinitsky and Barenholz, 1978) while TMA-DPH is

restricted to the hydrophilic surface (Kuhry et al., 1983). Consequently, only interactions altering the physical state of lipids in these regions will be detected. In contrast, in non-metabolising cell systems, K^+ release will occur as a result of any interaction that alters cytoplasmic membrane permeability. Moreover, accumulation of organic compounds within membranes is not always accompanied by changes in membrane fluidity (Engelke et al., 1996).

A linear relationship existed between NaCl concentration and TBT and TPT concentrations that resulted in 50 and 20% inhibition of cell viability, respectively (Fig 3.6.9a). Organotin toxicity was related to lipophilicity as indicated by the linear correlation between D_{ow} values and inhibition of cell viability (Fig. 3.6.9b). As the K_{ow} values of TBTCl and TPTCl, 4.76 and 4.19, respectively, are higher than that of TBTOH (4.10) and TPTOH (3.53), greater overall lipophilicity results as the fraction of chloride species increases. The linear relationship between D_{ow} and cell death suggests that TBT and TPT toxic effects were related to interactions with the cell membrane. Consequently, the difference in toxicity between chloride and hydroxide species can be attributed to the lipophilic properties of the compounds.

The limited reports on the influence of NaCl on organotin uptake and toxicity have suggested that organotin interactions are reduced in the presence of NaCl (Cooney et al., 1989; Laurence et al., 1989; Avery et al., 1993). This reduction in toxicity has been attributed to three main causes: (i) Na^+ may reduce interaction of organotins with the cell surface by competing for binding sites or interacting with the compound itself (Cooney et al., 1989), (ii) the membrane lipid composition may be altered, making the cells more resistant to membrane-acting compounds (Cooney et al., 1989) and (iii) Cl^- can inhibit the solubility of organotin compounds by association with the cation to form covalent organotin chloride (Blunden et al., 1984). For the experiments described here, these reasons can be discounted. Firstly, Na^+ did not inhibit uptake of either TBT or TPT so competition for binding sites did not occur and alteration in toxicity was not due to different uptake levels. Secondly, cells were only exposed to NaCl for a short time period and in the absence of a carbon source so any NaCl-related membrane changes could be discounted. The similarity between anisotropy values after exposure of cells to 0, 50 and 500 mM NaCl, confirmed the lack of change in membrane fluidity. Finally,

the maximum concentrations of organotins examined were selected so as not to exceed the solubility limit predetermined for 500 mM NaCl (Section 4.1.2). Alteration in toxicity was concluded to be due to a different factor, namely the difference in toxic effects of the organotin species.

The variation in TBT and TPT toxicity with speciation is consistent with that proposed for inorganic and methymercury. The speciation of mercuric compounds, as influenced by pH and NaCl concentration governs toxicity in the diatom, *Thalassiosira weissflogii* (Mason et al., 1996). The effect of mercury compounds on the growth rate of *T. weissflogii* increased with the fraction of neutral chloride species ($HgCl_2$ and CH_3HgCl) present in solution. For example, at 10‰ salinity, with 23% of inorganic mercury present as $HgCl_2$, phytoplankton growth was completely inhibited, while no inhibition was detected at 20‰ salinity, with the $HgCl_2$ fraction reduced to 7.5%.

Organotins are usually present at low concentrations in polluted environments (Section 1.1), at levels well below solubility limits. In this case, the influence of NaCl on toxicity is of utmost significance. Contrary to previous reports, the present results demonstrate that toxicity of TBT and TPT was enhanced with increasing NaCl concentration. This increased toxicity was attributed mainly to the formation of chloride species with toxicity of the organotin species increasing in the order $R_3Sn^+ < R_3SnOH < R_3SnCl$. Consequently, the toxicity of organotins and their interactions with microoganisms will be governed by the chemical properties of the environment. In seawater (pH~8, ionic strength~0.5 M) 93% of the TBT in solution is predicted to be TBTOH with 2-3% TBTCl and 4-5% TBT^+ and TPT is predicted to be present almost exclusively as TPTOH (>99%) (Arnold et al., 1997). However, TPT and TBT pollution is also associated with rivers and estuaries where pH may vary below pH 5 in heavily polluted conditions and salinity will have to be taken into account. Under these conditions, organotin toxicity will be heavily influenced by the species present in solution.

4.7 Relationship between organotin toxicity and lipophilicity

The Cl^- and pH-dependent speciation of organotins has consequences for the partitioning between aqueous and organic phases and plays a pivotal role in the physicochemical basis of their toxicity to microorgansisms. Although the theoretical basis is given (Meador, 1996), there is, however, a lack of experimental data on the influence of chemical speciation on organotin toxicity to microorganisms. From the results presented here it is clear that pH and Cl^- concentration influences organotin-yeast interactions and this relationship is based on the organotin species present in solution. To further elucidate the relationship between organotin speciation and toxicity, the variation in toxicity with lipophilicity, as indicated by D_{ow} values was examined.

The D_{ow} values of TBT and TPT under the different pH and NaCl conditions were calculated. Organotin toxicity was determined as IC_{50} and IC_{20} for TBT and TPT, respectively, corresponding to each experimental condition. There was a linear correlation between TBT D_{ow} and IC_{50} ($r^2 = 0.860$) and TPT D_{ow} values and IC_{20} ($r^2 = 0.975$) (Fig 3.7.1). This confirmed that there was a direct relationship between toxicity and the overall lipophilicity of the species present in solution. For both compounds there was over a 30-fold difference in IC values between the highest and lowest D_{ow}. Lipophilicity, as reflected by K_{ow} values, of structurally distinct di- and trisubstituted organotins, with up to six carbon atoms per substituent also correlates with toxicity toward fish cell cultures (Bruschweiler et al., 1995). Other physiochemical parameters such as total molecular surface area and steric characteristics have been correlated with toxicity towards aquatic organisms including algae and bacteria (Wong et al., 1982; Eng et al., 1991) and mudcrabs (Laughlin et al., 1985). However, this is the first time that the actual D_{ow} of an individual compound, as determined by the species present in solution, has been correlated with toxicity.

The overall relationship between organotin toxicity and lipophilicity was examined further by combining the results for both compounds. IC_{20} values were calculated from all TBT data and combined with TPT IC_{20} results. Similarly, TPT IC_{50} values, which were only available for NaCl-containing systems, were combined with all TBT IC_{50} data. There was a clear correlation between inhibitory concentrations and D_{ow},

as determined for both IC_{20} and IC_{50} (Fig 3.7.2). For the compounds examined individually, the increase in D_{ow} values is indicative of the greater presence of neutral species in solution so increased toxicity may be due to either the D_{ow} or toxicity differences in the actual species. However, consideration of the pooled D_{ow} data for both compounds also shows that a linear relationship between increasing toxicity and lipophilicity exists. It is evident that toxicity of TBT and TPT is a function of the hydrophobicity of the compounds in solution. This relationship may be extended to include other organometal compounds, provided that there is no solubility problem.

There was no correlation between D_{ow} and organotin uptake. As discussed previously (Section 4.5) the D_{ow} model does not take biosorption of cationic organotins to the cell surface into account. Also, the concentrations examined here were well below those at which saturation would occur and under most conditions no variation in uptake levels was observed. However, the correlation of toxicity with lipophilicity indicates that toxic effects were dependent on dissolution in cell membranes. Uptake of lipophilic metal complexes by diffusion mechanisms has been described elsewhere. The transport of mercury across biological membranes is attributed to rapid diffusion of neutral species with no facilitated transport mechanisms being documented (Pelletier, 1996). Similarly, passive uptake of neutral chloride complexes is the principal accumulation route of both methylmercury and inorganic mercury in phytoplankton (Mason et al., 1996). Uptake of lipophilic, organic-Cu metal complexes, $Cu(DDC)_2^0$ (diethyldithiocarbamate-Cu complex) and $Cu(Ox)_2^0$ (8-hydroxyquinoline-Cu complex) by *T. weissflogii* occurs by diffusion (Phinney and Bruland, 1994). In this case, two mechanisms are proposed to be involved. First, the lipophilic organic metal complex may partition into the cytoplasmic membrane, diffuse across the lipid bilayer into the cytoplasm and become distributed among the cellular compartments as a complex. Secondly, once the compound has diffused into the cytoplasm, the metal may dissociate from the chelate and bind to intracellular binding sites. Similarly, mercury accumulation and toxicity depends on the reactivity of each form of mercury with intracellular ligands in addition to passive diffusion of hydrophobic species across the cell membrane (Mason et al., 1996). The lipophilicity of these metal complexes, as indicated by K_{ow} is of the same magnitude to those of TBTOH, TBTCl, TPTOH and TPTCl. The K_{ow} values

of $HgCl_2$ and CH_3HgCl are estimated to be 3.3 and 1.7, respectively (Mason et al., 1996), while K_{ow} values of $Cu(DDC)_2^0$ and $Cu(Ox)_2^0$ were 2.8 and 2.6, respectively. These findings also support the conclusion that diffusion and lipophilic interactions play a significant role in TBT and TPT uptake mechanisms.

CHAPTER 5

CONCLUSION

Chapter 5: Conclusion

The general objectives of this research were to determine the principal interaction mechanisms between organotins and yeast. Initially, the uptake and toxicity of inorganic tin and the triorganotins, TBT and TMT at pH 5.5 were compared. In order to assess removal of these compounds from solution at low, environmentally significant concentrations, a flow injection HGAAS system was developed for tin analysis. Toxicity of the compounds was assessed in terms of cell viability and membrane damaging effects. Membrane integrity was analysed by K^+ release and fluorescence anisotropy of DPH and TMA-DPH. Generally, these fluorescent probes are used to monitor the effects of organotins on model lipid membranes (Ambrosini et al., 1991a and Ambrosini et al., 1996) and this is the first time that they have been used to assess organotin interactions with yeast cells. The importance of cell wall binding was assessed by comparison of intact cells and protoplasts.

The present study demonstrates that while both Sn(IV) and TBT are taken up by *C. maltosa*, the uptake mechanisms are different. Sn(IV) uptake was consistent with cationic interactions with functional groups on the cell surface and resulted in no K^+ leakage or viability loss. TMT, at concentrations up to 100 μM, did not become cell associated and no interactions were apparent between pH 3.5 and 7.5. TBT uptake levels were similar for intact cells and protoplasts and accumulation resulted in toxicity at all concentrations examined. Uptake resulted in extensive K^+ release and alteration in membrane fluidity, implicating lipophilic interactions as an uptake mechanism. At low initial concentrations all of the TBT may be removed from solution by microbial uptake. As microorganisms are at the base of the food web, direct accumulation in higher organisms may ensue.

To further examine the significance of lipophilic interactions, the influence of external pH and NaCl concentration on uptake and toxicity of TBT and TPT was investigated. Generally, a reduction in organotin-microorganism interactions with increasing pH or NaCl concentration has been suggested (Avery et al., 1993; Cooney et al., 1989; Laurence et al., 1989). However, in these reports, organotin insolubility, leading to a reduction in uptake levels and toxicity, can not be discounted. Here, TBT

and TPT concentrations were chosen so that they were below predetermined solubility levels.

Organotin interactions varied considerably between pH 3.5 and 7.5. Uptake and toxicity of TBT and TPT was lowest at pH 3.5. This was due to protonation of cell wall binding sites, resulting in reduction in R_3Sn^+ biosorption. In both cases, K^+ release and membrane fluidity changes were negligible, confirming the absence of membrane interactions. TPT toxicity increased with pH and was associated with the presence of the neutral hydroxide species (TPTOH) in solution. Effects on membrane integrity were also enhanced at the higher pH with a decrease in membrane fluidity of lipids in the hydrophobic tail region occurring at pH 5.5 and 7.5. The relationship between TBT toxicity and pH was not as simple. TBT uptake was reduced only at pH 3.5 with similar uptake levels at the higher pH. The effect of TBT on cell viability increased from pH 4.5 to 5.5 with very different membrane effects occurring. At the lower pH, TBT caused an increase in membrane fluidity at the surface of the cytoplasmic membrane. In contrast, at pH 5.5, there was an increase in DPH anisotropy, indicating a reduction in fluidity at the hydrophobic core of membrane lipids. However, at pH 7.5, TBT effects on membrane fluidity were not detected, with K^+ release levels less that at pH 5.5. It is proposed that TBTOH can traverse the cytoplasmic membrane, entering the cytosol, similar to the passive diffusion of organic metal complexes (Phinney and Bruland, 1994).

Organotin toxicity increased with NaCl concentration. This corresponded to the increasing fraction of the lipophilic R_3SnCl species in solution. The general toxicity of the individual organotin species increased in the order $R_3Sn^+ < R_3SnOH < R_3SnCl$. These results have implications for prediction of organotin behaviour in marine and estuarine systems. The chemistry of the aqueous environment will have a key role in determining organotin toxicity with the formation of cationic (R_3Sn^+) and neutral (R_3SnOH and R_3SnCl) species in solution.

Previous studies on TBT interactions neglect to take uptake by diffusion mechanisms into account, with cell wall biosorption proposed as the principal uptake mechanism. However, these results clearly demonstrate that lipophilic interactions play an essential role in organotin toxicity. Due to the low concentrations examined,

differences in uptake levels were not apparent. There was a linear correlation between organotin toxicity and compound lipophilicity, as indicated by D_{ow} values. This relationship existed for both TBT and TPT examined individually and also when the data for both compounds were pooled.

These results indicate that the physicochemical interactions between organotins and microorganisms will be predicted largely by the organotin species present in solution. Organotins have been detected in a wide variety of ecosystems, including both freshwater and estuarine water and sediment. Seawater consists of a number of ionic species including Cl^-, Na^+, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-} and HCO_3^-, while these ions are found to a lesser extent in estuaries. An understanding of the composition of the external environment and its influence on speciation is key to assessing organotin pollution as these ions will play a pivotal role in their interactions with microorganisms.

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