ORGANOLEAD COMPOUNDS
IN NATURAL SYSTEMS

by

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SUMMARY

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Most organolead compounds with alkyl groups attached to a central lead atom, are many times more toxic than inorganic lead compounds. The widespread use of tetraethyllead and tetramethyllead as antiknock agents in gasoline, and consequently their emission or discharge into the environment, is therefore of considerable importance.

It is thought that alkylleads in an estuarine environment were responsible for the death in 1979 of a large number of coastal birds. There are relatively little data on the fate, toxicity or accumulation of these compounds in natural systems, such as bivalve molluscs. These animals are an essential part of the diet of coastal birds and may have accumulated alkyllead to concentrations that were acutely toxic to the birds.

Data reported in this thesis indicate the approximate concentrations of the most environmentally abundant alkylleads that are toxic to two estuarine bivalves and two algae. It is observed that high concentrations may accumulate in bivalves, which may be acutely toxic to birds.

In addition, the relative toxicities of a number of alkyllead compounds are established in an attempt to resolve conflicting reports of tetraethyllead toxicity in the literature. Our results suggest that that tetraethyllead is not toxic in its pure form, but that the ionic triethyllead and trimethyllead are highly toxic.

Evidence is presented suggesting that triethyllead may be adsorbed onto tetraetyllead molecules both in solution and in cytoplasm causing a reduction in overall toxicity. It is also likely that certain unicellular algal cells may be capable of metabolising alkyllead compounds via dealkylating mechanisms.

Keywords: Alkyllead, Bivalves, Unicellular algae, Toxicity.

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CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1. General Properties and Definition

The alkylleads commonly encountered in the environment are the methyl and ethyl derivatives, which are used as antiknock fluids. Most environmental studies, including this one, are mainly concerned with these compounds. In the organoleads the covalently bound lead atom is tetravalent unlike the divalent form found in inorganic compounds where ionic bonds occur. Thus alkyllead compounds have the following formula:

$$R_{4-n}\ Pb\ X_n$$

where R= alkyl group, X= anion e.g. Cl, n= 2 to 0.

For example, the ethyl lead series could be:

$$Et_4Pb \qquad Et_3PbCl \qquad Et_2PbCl_2$$

Monoalkyllead compounds are usually unstable and have short lifetimes, unless the alkyl substituent is very large[1].

Tetraalkylleads (TAL) are highly insoluble in water, whether it be fresh or marine (solubilities are: TEL = 0.10 - 0.19mg l^{-1}, TML = 15.0mg l^{-1}[2,3] N.B. Concentrations of organolead species are expressed on the basis of the lead content only, throughout this thesis), but are soluble in most organic solvents. The compounds are predominantly colourless liquids at room temperature with high volatility.

The trialkyllead and dialkyllead compounds are considerably more soluble in water and other polar solvents than the TALs, and they also have some solubility in organic solvents. They are white solids at room temperature.

The covalent nature of an alkyllead series (e.g. ethyl) increases with the degree of alkylation, so that dialkyllead is the most ionic of the commonly encountered compounds. Indeed dialkylleads possess many similar properties to inorganic lead compounds. Long-term stability of alkylleads

varies with degree of alkylation. Generally, the species will decompose at increasing rates in the following manner:

$$R_3Pb^+ < R_2Pb^{++} < R_4Pb$$

Decomposition can occur through dealkylation in aqueous systems. For example:

$$TEL \rightarrow Et_3Pb^+ \rightarrow Et_2Pb^{++} \rightarrow Pb^{++}$$

The major process is photolytic degradation involving free radical mechanisms, and under natural illumination very rapid decomposition of TAL may proceed. For example, the rates of degradation of vapour phase tetraethyllead (TEL) and tetramethyllead (TML), two of the most common environmentally occurring TAL compounds, have been investigated by Harrison and Laxen[4]. Photolytic decay rates were 8% hr^{-1} TML, and 31% hr^{-1} TEL during bright sunlight at a solar zenith, at 40°C. Decomposition also occurs in the dark, although the rates are much slower and the mechanism is probably ionic[5,6].

1.2. Uses of Organolead Compounds

Alkyllead compounds are primarily produced as gasoline additives to suppress knock in the internal combustion engine. Commercial production of TEL first began in the early 1920's in the U.S.A. Although known to be toxic, several human deaths occurred through handling and manufacture of antiknock compound[7]. These fatalities prompted a temporary ban on production, during which time safer, more hygenic and protective measures were developed.

Shortly after the start of commercial production it was discovered that the addition of dichloroethane and dibromoethane, as scavengers, to the organolead compound extended the lifetime of an engine. This is because lead oxide formed during combustion is converted to volatile lead halogenides which, unlike the former, do not deposit within the engine[7].

In 1960 TML was introduced into antiknock fluid because it has slightly more beneficial properties than TEL under certain circumstances, i.e. higher volatility and antiknocking ability[8]. Mixed ethyl-methyl compounds ($EtMe_3Pb$, Et_2Me_2Pb , Et_3MePb) have since been used, the exact nature of the mix being governed by the type of fuel in which they are intended to operate.

lead not alkyl lead ?

Concern about the effect of alkyllead compounds on human health has pressurised successive governments in many countries, including Britain, to reduce or ban the addition of lead alkyls to gasoline. This is in spite of the failure to find a suitable cost effective replacement for leaded antiknock compounds after years of intense research. Nations such as Australia, Japan, New Zealand, Switzerland and certain Scandinavian countries have, in the most part undertaken to further reduce leaded fuel use in new motor vehicles, although in some cases e.g. U.S.A., this was not on health grounds. In 1972 the lead content of fuel in the U.K. was 0.84g l^{-1}. This was reduced to 0.45g l^{-1} by the end of 1978 and to 0.40g l^{-1} in 1981[9]. In 1978 a European Community Directive outlined the upper and lower limits of lead in petrol as 0.4 and 0.15g l^{-1}[10]. At lead concentrations below 0.15g l^{-1} present Western European octane quality cannot be maintained[11]. In 1983 the 9th Report of the Royal Commission on Environmental Pollution[11] strongly recommended the phasing out of leaded petrol and suggested that all new motor vehicles could be manufactured to run on unleaded fuel by 1990. Although this would require the revision of the 1978 Directive[10] for minimum lead limits, the proposals were included in the elected Conservative Government's manifesto later that year.

Other uses of alkyllead compounds are small in comparison with antiknock compounds. These uses are predominantly based on the toxicological properties of a variety of compounds. Tributyl and triphenyl lead

acetates are both effective biocides incorporated into marine antifouling paints. The compounds prevent sessile organisms from attaching, and thereby reduce fuel losses due to increased resistance to passage and maneuverability.

Tributyllead acetate also shows antifungal activity in emulsion paints and is more effective than organotin or organomercuric compounds. Triphenyllead acetate is also effective in combating Bilharzia, a schistosome infection. The compound eliminates the parisite vectors present in tropical gastropods. A similar function was proposed for alkylleads, to remove tapeworms in sheep. Although some success was reported in adults, the concentrations used were toxic to lambs. Other potential biological uses are as wood and textile preservatives, e.g. against marine wood borers, and in cotton production.

The catalytic properties of organolead compounds make them useful in polymerization reactions such as in the manufacture of polythene. They also possess stabilizing properties which are employed in heat stabilization of polyvinyl chloride. Another non-biological use is as lubricant additives, some organolead compounds having very efficient antiwear applications. The various uses of organoleads are reviewed in greater detail in the literature[1,12].

1.3. Environmental Contamination

Relatively high concentrations of lead occur naturally in the environment. Background soil levels may range from 2 to 200ppm[13]. In comparison both the occurrence and usage of organolead are small but nevertheless significant. Man-made alkyllead contamination of the environment may arise from several sources, most of these reflecting the greatest use of the compounds as antiknock agents. The most widespread and frequent must be that from automobile exhaust fumes. Combustion of gasoline is incomplete

especially in cold engines, resulting in atmospheric emissions of TEL, TML and the decomposition products[14]. The fraction of unburnt lead is relatively small compared with the initial input concentration, but in urban areas or near heavily used roads, atmospheric loading can be high. From the several studies of exhaust emissions, a rough figure of 0.2% of input lead is emitted as TAL during steady driving at constant speed, however there is great variation between different cars[15].

The fate of emissions from exhausts depends upon particle size, amongst other factors. Larger particles are more likely to fall to ground, whereas finer particles and aerosols will exist in the ambient atmosphere for greater periods. TAL compounds breakdown rapidly in the atmosphere in a similar manner to hydrocarbons in general[4]. Reactions with ozone and hydroxyl groups occur most commonly, so that in less polluted atmospheres breakdown is slower because of lower hydroxyl concentrations. However, photolytic decomposition accounts for most alkyllead breakdown. Stability and persistence is therefore greater during night-time[4]. A study of leaded gasoline consumption in Los Angeles concluded that, with the aid of a mathematical model, 20% to 25% of aerosols from exhausts ended up in the oceans, with 75% of input lead exhausted, 3.8% of this consisting of organolead vapour[16]. Even with photolytic decomposition resulting in a 21% to 29% reduction in TML hr^{-1} and a 88% to 93% reduction in TEL hr^{-1}, some TAL may still enter aquatic systems[4].

Loss of leaded gasoline during handling operations, especially at filling stations due to evaporation accounts for approximately 1.3% of the TAL in gasoline entering the atmosphere[16]. This is predominantly due to the relatively high vapour pressures of these compounds. The values for TEL and TML at 20°C are 0.26 and 23.7 mm Hg respectively[17]. Combined with the loss in exhaust fumes, about 7000 tons of TAL was emitted into the atmosphere from filling stations and vehicles in the Western World

in 1975[16].

Contamination from antiknock production sites and refineries processing leaded gasoline should be minimised by strict on site process controlling. However, it was estimated that in the U.S.A. in 1970, 11% of the total lead emitted was from manufacturing plants. The proportion of alkyllead in this estimation was unknown[18]. Such emissions may be atmospheric, or as effluent discharges into the aquatic environment.

Other sources of aquatic alkyllead pollution may arise from minor spills during handling, but more seriously from major loss of antiknock compound as in the case of the Yugoslavian ship 'Cavtat'. In 1974 a collision involving two vessels resulted in the sinking of the 'Cavtat' 3.5 miles off the Otranto Cape, Southern Italy, in 94 meters of water[19]. The ship was transporting 900 drums containing some 325 tons of TEL and TML. A salvage operation took place three years later to retrieve the load, because of the severe corrosion many drums had sustained. All but 7% of the drums were recovered, preventing severe long-term pollution. Two previous accidents involving considerable amounts of antiknock occurred near Trinidad in 1961 and Cape Town in 1966, but no studies were undertaken to assess the damage to the environment.

Contamination of the aquatic environment by TAL will result mainly from accidental release into water, as was the case for the 'Cavtat'. This is due partly to the rapid degradation chemistry of the compounds to form inorganic lead in the atmosphere. However, alkylation or more specifically, methylation of lead should also be considered. If methylation occurred at a significant rate, either by biological or chemical processes, then alkyllead pollution would be a major concern because of the continuous input of inorganic lead in sediment systems. Anaerobic microorganisms from lake sediments have been claimed

to possess the ability to methylate several lead compounds (organic and inorganic) including lead chloride, lead nitrate and trimethyllead acetate, to TML[20-22]. Other circumstantial evidence for biomethylation exists from high alkyllead concentrations above coastal mudflats at Morecombe Bay, Cumbria, U.K.[23]. Other workers have shown that chemical methylation can be used to explain the results obtained from 'biologically produced alkyllead' experiments[24-26], and the evidence we have so far would suggest that naturally occurring methylation is not an environmental hazard.

1.4. Toxicity of Organolead Compounds

Considering the widespread use of organolead compounds, especially TML and TEL, there has been relatively little work undertaken to study their effects on organisms. Several reviews of alkyllead toxicity are available covering most of the literature to date[1,15,27,28]. TEL, TML, their trialkyl and dialkyl derivatives and inorganic lead have been studied in sufficient detail to enable some generalization, but information concerning other organolead compounds is more scant.

Information about the effects on man has mostly resulted from studies of accidental poisoning during industrial handling of the antiknock compound[29-31]. A few isolated non-industrial incidents have also occurred including cases of gasoline sniffing[32-34], or mistaken usage of the compounds[35]. Thus the majority of data involving humans concerns severe poisoning, sometimes resulting in death. General low level alkyllead concentrations and their effects on humans, in the short and long term, have received almost no investigation.

The majority of experimental data concerning mammals has arisen from work on rats, mice, dogs and to a lesser extent rabbits. TAL compounds are rapidly absorbed through the skin of mammals primarily due to their high lipid solubility[36], but these are generally less toxic than their

trialkyl analogues. From studies on rats, TEL has been shown to be metabolized predominantly in the liver microsomes via an enzymatic pathway to $Et_3 Pb^+$ [37]. Dealkylation also occurs in kidney homogenates and brain brei and brain slices of rats and rabbits, but to a much smaller degree. No evidence of TEL metabolism in blood, spleen or muscle tissue has been obtained[38]. The actions of individual compounds of the ethyl series on rat brain brei and slices are different[37]. Lactate usage by brain brei and glucose oxidation by brain slices are both inhibited by Et_3Pb^+, but TEL and lead acetate showed no effect. Diethyllead acted in a different manner to triethyllead and its toxicity was lower. Glucose oxidation of kidney or liver slices was not inhibited by Et_3Pb^+ suggesting a selective toxic action in the nervous system.

Deaths by TEL and Et_3Pb^+ poisoning are identical, due to the action of Et_3Pb^+ in both cases[37]. Indeed very little TEL is found in the body shortly after death because of its rapid conversion to lower analogues[37-39]. Several workers have determined the LD_{50} for various lead alkyls in rats showing that the trialkyllead compounds are generally more toxic than TAL (Table 1.1.). In rats TEL is approximately 10 times more toxic than TML, possibly due to the faster rate of conversion of TEL to Et_3Pb^+ than TML to Me_3Pb^+ [40]. Data for humans and rabbits also suggests that TEL is more toxic, however mice and dogs appear to be more susceptible to TML[40-42].

Little is known about the biochemical mechanisms that affect the central nervous system and alter cell morphology as a result of alkyllead poisoning. Many investigations suggest that the brain is the critical organ. Acute cases of poisoning show certain neurological symptoms common to TAL poisoning, such as hyperactivity, aggression, convulsions and coma[42,43]. Other neurological symptoms can be seen in chronic poisoning[44]. There is evidence that triethyllead can alter the

TABLE 1.1 LD_{50} VALUES FOR RATS EXPOSED TO ORGANOLEAD COMPOUNDS. (FROM REF 15.)

IG. – Intragastric IV. – Intravenous
IP. – Intraperitoneal

Compound	Administration	LD_{50} (mgPb/Kg)	References
Et_4Pb	IG.	14	Schroederer 1972a
"	IG.	12	" 1972b
"		~20	Springman 1963
"	IV.	15	Cremer 1959
"		14	Magistretti 1963
"	IP.	15	
Et_3PbCl	IG.	~20	Springman 1963
"	IV.	11	Cremer 1959
Et_2PbOAc_2	IG.	130	Gras 1968
Et_3PbCl		~120	Springman 1963
Me_4Pb	IG.	80	Springman 1963
"	IV.	88	Magistretti 1963
"	IP.	90	
Me_3PbCl	IG.	<36	Springman 1963
Me_2PbOAc_2		120	Gras 1968
Pb^{++}	IV.	~150	

References.

distribution of anions resulting in changes in biochemical function of nerve cells[15]. Swelling of cells occurs after cessation of the actively maintained electrolyte balance. Other neurological changes have been investigated and it can generally be concluded that serious, though non-specific damage to the nervous system occurs in alkyllead poisoning[15,45].

Experiments involving a purified enzyme preparation of horse serum cholinesterase have shown that $Et_3 Pb^+$ deactivates the enzyme[46]. On introduction of pyridine-2-aldoxime methiodide (PAM) and pralidoxime methylsulfate, up to 80% of the enzymes original activity can be restored. This leads to the possibility of using these compounds as potential antidotes for TEL poisoning.

The mutagenic, carcinogenic and teratogenic effects of alkyllead compounds have received little investigation. From the studies to date, there is a suspicion of TEL mutagenic activity, but further elucidation is required, as other work is not in full agreement[47-49].

The majority of other investigations into alkyllead toxicity are concerned with aquatic organisms. The effects of inorganic lead are fairly well established for both freshwater and marine organisms. However, there is considerably less information concerning the effects of alkyllead. Considering the potential for pollution of coastal waters by spillages, effluents and atmospheric deposition, it would seem important to assess the impact of these factors, but very little work has so far been done.

Similar trends found in mammalian studies are apparent when the data for aquatic species are considered. For example, bioassays on acute toxicity of commercial TEL and TML on various marine groups clearly show that TEL is toxic to a greater degree (Table 1.2.)[50]. An

TABLE 1.2 COMMERCIAL TEL AND TML 48HOUR LC_{50} VALUES FOR SOME AQUATIC ORGANISMS. (FROM REF. 50)

Compound	Organism	0% Effect (ppb)	50% Effect (ppb)	100% Effect (ppb)
TML-CB	bacteria	900	1900	4500
	alga	450	1650	4500
	crustacean	180	250	675
	fish	45	100	250
TEL-CB	bacteria	80	200	2000
	alga	100	150	300
	crustacean	25	85	260
	fish	10	65	130

extensive study involving four marine phyla: algae, mollusca, crustacea and fish, concluded that the toxicities of TEL, TML, trialkyllead and inorganic lead can generally be stated as:

$$TEL > TML \gg Et_3PbCl > Me_3PbCl \gg Pb^{++} \quad (51)$$

The TAL compounds were about 70 times more toxic than trialkylleads which, in turn, were about 50 times more toxic than inorganic lead. Thus toxicity appears to increase with the degree of alkylation, although there are exceptions to this trend in both aquatic organisms and mammals. Aquatic toxicity is discussed in greater detail in Chapter 7.

1.5. The Mersey Estuary Bird Mortality Incident

During the autumn of 1979 several dead or dying coastal birds were discovered on certain reaches of the Mersey estuary[52]. The numbers of birds found were unusually high and during the following winter period these numbers rose to levels indicative of some major environmental perturbation. The majority of affected species were waders, which feed predominantly on the invertebrate fauna inhabiting the mud and sand banks.

The main species (of 26 in total) were dunlin (Calidris alpina), blackheaded gull (Larus ridibundus), redshank (Tringa totanus), teal (Anas crecca) and several other waders, wildfowl and gulls. Dunlin were most affected with about 1300 dead birds found. A total of approximately 2400 mortalities were recorded over the period, most being reported in October. These mortalities were greater than any previously recorded incident in the estuary, and they occurred mainly in a few areas in the centre of the estuary.

The behaviour of the dying birds suggested that they may have been poisoned. Symptoms included constant tremor, flightlessness, disorientation and an incapability to feed. Several dead birds were examined and analysed to determine any common cause of death. No consistent cause of death was found, although elevated lead concentrations were present. Concentrations in dunlin liver ranged from 6 to 36 mg kg^{-1} wet weight[52]. There is very little data covering normal lead concentrations in coastal birds, making interpretation of the dunlin analyses difficult. However, the literature available suggests that concentrations of approximately 4 mg kg^{-1} wet weight are on the high side of normal, and at concentrations above 10 mg kg^{-1} toxic effects should be expected[52]. The levels in Mersey dunlins were therefore most likely to have been highly toxic and were probably the cause of death.

Speciation of the individual lead compounds accumulated revealed considerable alkyllead content, although negligible TAL was found. 30% to 70% of the lead was in the trialkyl form, but very little dialkyllead was present.

The source of this alkyllead must, in all probability, have been the food consumed by the birds. The majority of birds died in areas of limited invertebrate diversity, although these few species, mainly small bivalve molluscs and polychaete worms, occur in high densities. Analyses of Macoma balthica, one of the most abundant invertebrates, revealed trialkyllead concentrations of approximately 1.0 mg kg^{-1}. Based on average food intakes, birds could have been taking in up to 2.0 mg trialkyllead per kg dry weight per day[52].

It appears obvious that the large numbers of bird mortalities recorded in 1979 were the consequence of trialkyllead poisoning as a result of consumption of contaminated invertebrates inhabiting certain reaches of the estuary. As the majority of the birds affected were winter visitors, the relatively sudden mortalities in the autumn were not surprising. However, why did such an incident not occur to the same extent prior to 1979 ? One possible explanation might lie in a combination of factors at work in the estuary. 1979 may have been a highly productive year for species such as Macoma balthica, so that there were more than usual for the birds to feed upon. Individual birds could then have consumed toxic amounts of alkyllead by feeding almost exclusively on the molluscs which had accumulated alkyllead. The exact causes of this singular incident are complex and may never be thoroughly explained. The present lack of understanding of the interactions between alkyllead compounds and the natural environment is clearly highlighted by the 1979 mortality. In an attempt to improve this understanding, investigations into the effects of alkyllead on bivalves, including Macoma balthica, and

unicellular algae were undertaken, and the results of these investigations are reported in this thesis.

CHAPTER TWO

ANALYTICAL METHODS

ANALYTICAL METHODS

2.1. Introduction

The aim of this study was to investigate the toxicity and accumulation of several different alkyllead species. In many cases information about the occurrence and concentration of more than one alkyllead species was required from a single experimental sample. The low concentrations of lead species present in such samples, often in the parts per billion range ($\mu g\ l^{-1}$), required that the analytical methods used be very sensitive.

It is not surprising, therefore, that in many cases several techniques were needed to obtain all of the information required. The analysis also had to be capable of avoiding interference from other metals or trace substances present in low or background concentrations and therefore had to be extremely specific and accurate.

Due to the nature of this study qualitative analysis fulfilled only a minor requirement. Almost all of the analytical procedures used yielded quantitative results. The majority of analyses were carried out, after any pretreatments, using flameless atomic absorption spectroscopy (AAS), anodic stripping voltammetry (ASV) and GLC-mass spectroscopy (GLC-MS)

2.2. Determination of Alkyllead Compounds

There is extensive literature on the determination of organolead compounds because of the commercial importance of tetraalkyllead (TAL) and the need to monitor ambient levels of lead alkyls in antiknock manufacturing plants. The various analytical procedures have been reviewed by Shapiro and Frey[1] and Compton[53].

2.3. Qualitative Analysis

The widely used analytical techniques, namely infra-red and nuclear

magnetic resonance spectroscopy and elemental analysis, were employed to ascertain the identity of various reaction products during the attempted synthesis of trihexyllead chloride.

Infra-red spectroscopic analysis yields information about the types of molecular groups present in a compound. Nuclear magnetic resonance spectra give information about the chemical environment of protons present in the lead compounds. Elemental determinations for carbon, hydrogen and chlorine constituents of the compounds gives the percentage of these elements in the sample.

2.4. Quantitative Analysis

Most of the quantitative determination procedures employed in this study have been developed and used in routine sample analyses by the Associated Octel Company Limited. Thus, although there are many methods available, the ones used by us had been rigorously tried, tested and improved through use in both industrial and academic environments.

In many of the procedures available for alkyllead determination, pretreatment of the sample is necessary before the use of the various detector instruments. Pretreatment usually either extracts the specific compounds under investigation from otherwise interfering species, or chemically alters them to a form readily detectable.

Extraction procedures do not alter the chemical nature of the alkyllead species and are therefore less likely to suffer from contamination. Pretreatments involving chemical changes in the lead species are more susceptible to contamination, although these procedures may result in far lower detection limits.

2.4.1. Analysis of Aqueous Samples

Many methods can be employed to evaluate organolead species in water.

The procedure used most frequently by us relies upon the dealkylating ability of the reagent iodine monochloride in hydrochloric acid solution (ICl). This reagent converts tetraalkyl and trialkyl lead compounds to the dialkyl form. This is a far superior technique to the complete dealkylation to inorganic lead achieved by reaction with nitric acid/ bromine[54]. The seemingly ubiquitous contamination by inorganic lead is not a major problem in the former method.

Two agents, 4-(2-pyridylazo)-resorcinol (PAR) and diphenyltiocarbazone (dithizone), are used to combine with the alkyllead to form coloured complexes. Both complexes can be used for colorimetric determination [54,55], however by modifying the dithizone procedure to finish with AAS, lower detection limits are achieved[56].

2.4.1.1. Dithizone

Tetraalkylleads may initially be extracted from the sample by shaking with n-hexane. After separation, the organic phase may be treated in the same manner as the trialkyl and dialkyl containing aqueous phase, except that the hexane is removed before further organic additions are made. Alternatively the hexane layer may be analysed directly using GLC-MS.

Organolead is converted to dialkyllead by ICl and is then preferentially extracted from the buffered aqueous phase by dithizone in carbon tetrachloride. Inorganic lead is masked in the aqueous phase by ethylenediamine NNN'N' tetraacetic acid (EDTA). The organic phase is run off into polytetrafluroethylene (PTFE) tubes[25] and back-extracted with dilute nitric acid/hydrogen peroxide solution. Aliquots of the acid layer are injected into a graphite furnace and are analysed by AAS.

2.4.1.2. PAR

Using this technique water samples of organolead are determined

spectrophotometrically after the formation of the dialkyllead-PAR complex. Again ICl is used to dealkylate the higher alkylated lead compounds to the dialkyl, and n-hexane is used to remove any TAL present in the sample. This is analysed by AAS after dithizone extraction or by GLC-MS.

The PAR reagent is more specific than the dithizone in that it only combines with inorganic and dialkyllead and a few other common metals to form an orange coloured complex. Specificity is further enhanced when the reaction takes place in a sulphite buffer (pH 9) containing citrate and cyanide. These latter two groups reduce the interference from the trace metals, mercury and tin. Iron present in the sample may also interfere with the determination. Addition of ascorbic acid just before the PAR complexing stage usually eliminates this problem.

To prevent the complexing of PAR with inorganic lead the ligand cyclyhexanediamine NNN'N tetraacetic acid (CDTA) is added prior to PAR addition. Interference from calcium, magnesium and other common metal salts is also reduced by CDTA.

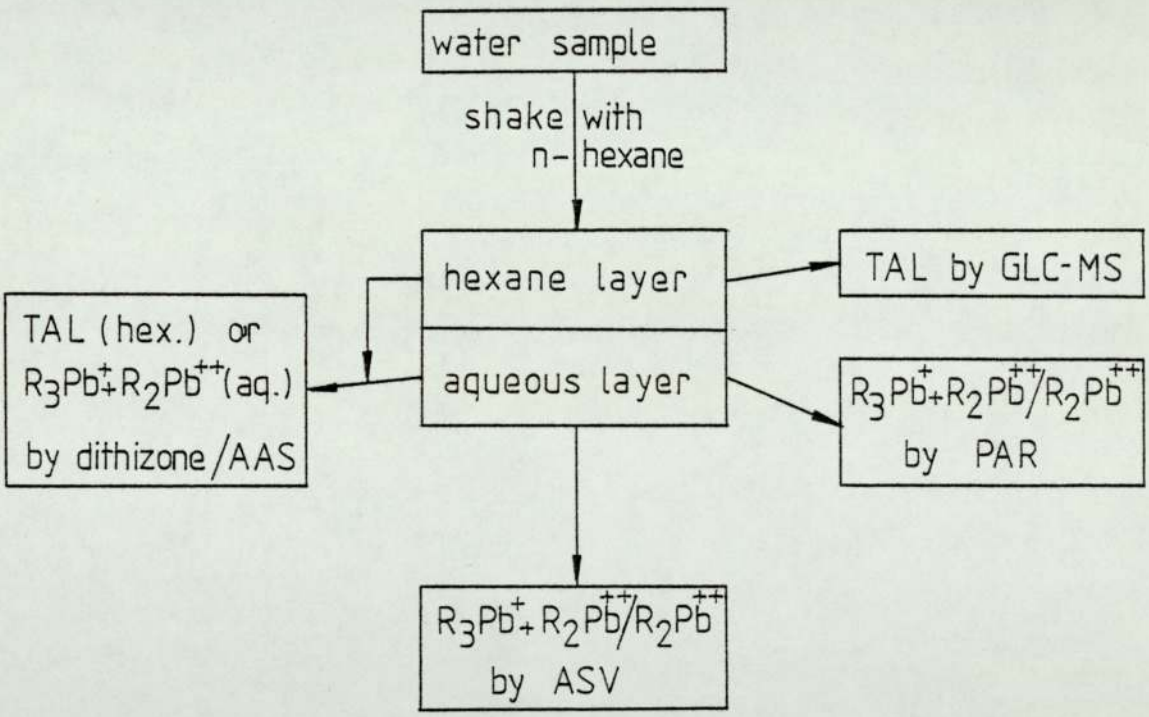
An aliquot of the sample containing the PAR complex is measured spectrophotometrically by determining the absorbance at 515 nm against a blank. The total trialkyl and dialkyl lead content is then calculated from a calibration curve.

Determination of the dialkyllead content of the sample is only achieved by adding the sample after the sulphite buffer has destroyed the ICl reagent. Thus the higher alkylleads are not dealkylated and only the indigenous dialkyllead is complexed with PAR. The difference between this value and that of the total dialkyl and trialkyl lead determination is a measure of the trialkyllead content of the sample.

Alternatively, when the instrumentation was available, water samples of

organoleads were determined by a polarographic technique[57]. This procedure is much preferred to the above methods because analyses can be carried out directly upon the aqueous solution and requires no extractions or chemical changes in the organic lead present. An overall scheme for the determination of alkyllead compounds in water samples can be seen in Fig.2.1.

FIG 2.1. THE DETERMINATION OF ALKYLLEAD COMPOUNDS IN AQUEOUS SAMPLES



2.4.2. Analysis of Biological Samples

Biological samples, whether molluscan tissue or unicellular algae were dealt with by one general procedure. Organic lead is extracted from the tissue into toluene, which is then back-extracted with dilute nitric acid for trialkyl/dialkyl lead determination, or analysed by GLC-MS for TAL.

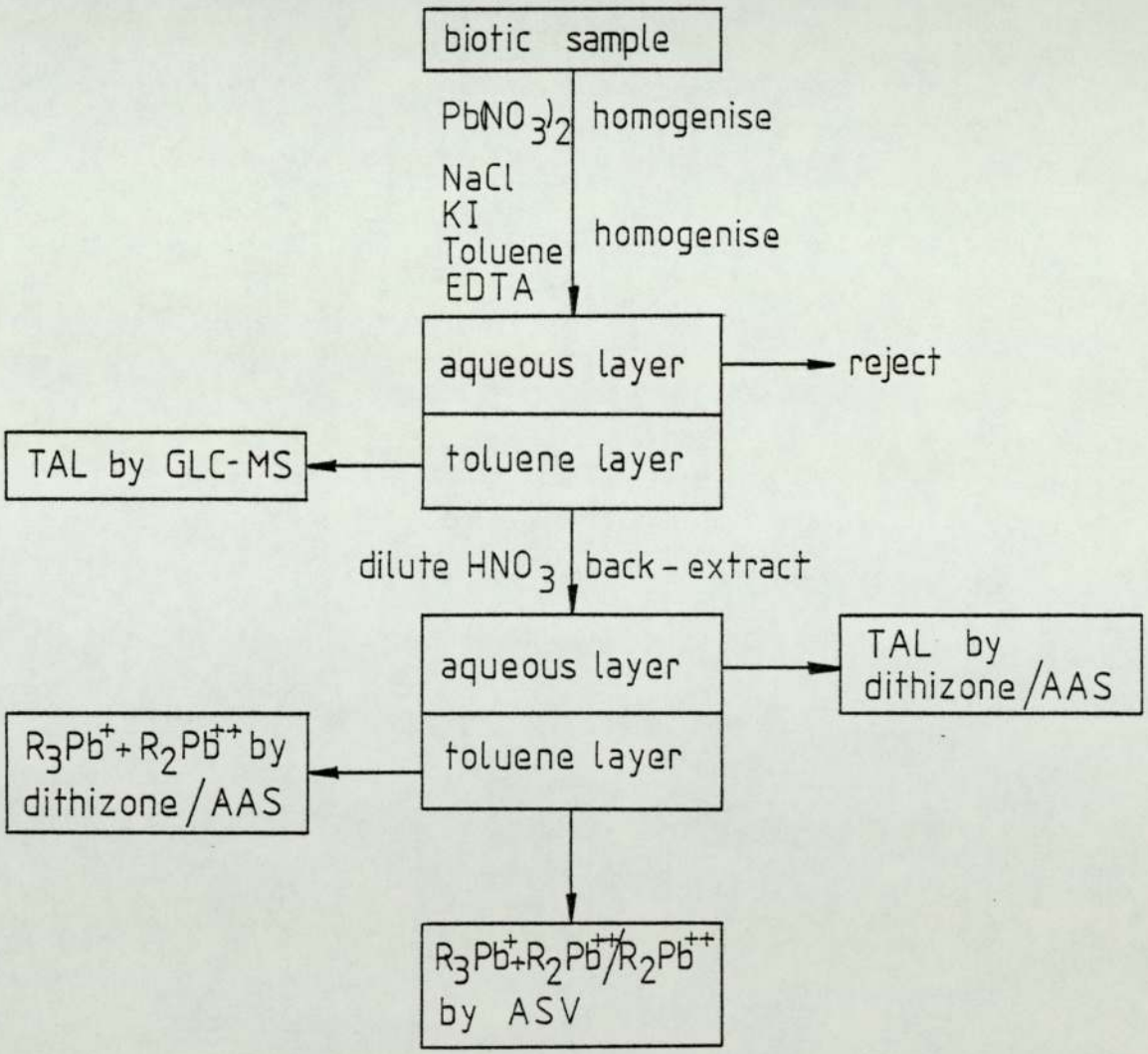
The sample is initially homogenised with inorganic lead in an iodide/chloride containing medium to aid the release of organolead from the tissue[55,57]. Sodium benzoate is also added in a controlled amount to improve the extraction of dialkyllead into toluene. However, it should be noted that too much sodium benzoate reduces the efficiency of trialkyllead extraction[57]. EDTA is added to complex inorganic lead and this remains mainly in the aqueous phase, whilst the organic lead is extracted into the toluene layer. A slight excess of EDTA is used to minimise the small transfer of inorganic lead into the toluene and subsequent phases.

An aliquot of the toluene layer is then taken, after centrifugation of the tissue-containing sample, and is analysed for TAL by GLC-MS without further pretreatment. Another aliquot of the toluene is back-extracted with dilute nitric acid. This isolates the ionic alkylleads from the TAL, which remains in the toluene. The analysis is then completed by ASV, enabling both trialkyl and dialkyl forms to be determined in the same sample.

When ASV facilities were not available, the analysis of trialkyl and dialkyl lead compounds was achieved using the dithizone extraction procedure on the dilute nitric acid layer. The remaining toluene layer could be treated similarly, for TAL determination, AAS being used in both cases as the finishing instrument.

Direct analysis of the acid layer, i.e. without dithizone extraction, cannot be used because of the presence of inorganic lead in the nitric acid. The ASV method of analysis is obviously preferred as sensitivity is lost during the multistage extraction and chemical alterations required for AAS determination. A general scheme for alkyllead determination in biological samples is given in Fig. 2.2.

FIG 2.2. THE DETERMINATION OF ALKYLLEAD COMPOUNDS IN BIOLOGICAL SAMPLES



2.5. Atomic Absorption Spectroscopy

AAS determines the total lead content of a sample. Any speciation of different lead compounds must be achieved by suitable pretreatment such as the dithizone extraction of dialkyllead. AAS works in the following general manner:

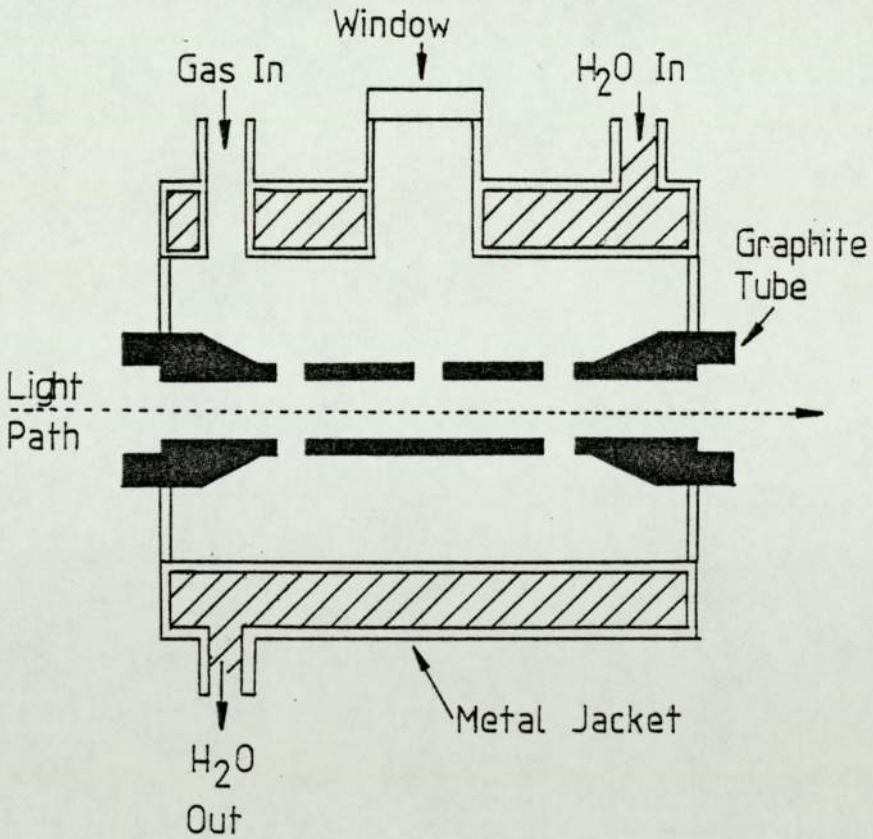
A hollow-cathode or cathodeless lamp emits a chopped exciting beam equivalent to the spectrum of the analyte (lead). This passes through a flame or heated graphite furnace where it is weakened due to absorbance by analyte atoms. The beam then passes to a monochromator, which isolates a specific single line of the analyte spectrum. A photocell amplifier and recording device then measure the intensity of the analyte line which is proportional to the concentration of analyte in the sample[58].

The hollow-cathode lamp is a low-pressure gaseous discharge tube, producing a low energy spectrum of the element under analysis. This is achieved by concentrating a beam of electrons onto a small area of the pure metal inside the lamp, exciting the atoms, and thus producing the specific emission spectrum required. The low pressure and temperature of the lamp aid the production of very narrow spectrum lines, which produces specific and efficient atomic absorption. Because the emission spectrum of the lamp is identical to that of the analyte, interference from other metals present in the sample is reduced. Interference from smoke and fume scattering is compensated for by a deuterium background corrector. A deuterium lamp produces a continuum beam which is inserted into the path of the cathode beam, to pass through the atomising device. The deuterium beam is then subtracted from the exciting beam signal.

Atomisation of the analyte (optimally to the ground state), is often achieved by a high temperature flame into which the sample is nebulized. This is a widely used technique, but has some disadvantages when

compared to the other methods incorporating heated flameless furnace atomisers[58]. The heated graphite furnace atomises a much greater proportion of the analyte than can be achieved by a flame. The atoms remain excited in the path of the beam for a longer time, so that peak measurements at maximum absorbance can be obtained over shorter time intervals. These factors contribute to the very high sensitivity of the furnace, being orders of magnitude greater than that of the flame atomization. Fig. 2.3. shows a typical electrically heated graphite furnace similar to the one used in this work. It is based on a design by Massmann[59].

FIG 2.3 GRAPHITE FURNACE



A 10 µl to 100 µl aliquot of the sample is pipetted into a graphite tube through a small hole beneath the removable window. Other holes in the tube allow circulation of an inert gas, usually argon, which prevents oxidation of the tube at the high temperature reached during atomisation. The tube is resistance heated when a current is supplied, and accurate temperature is maintained by varying the potential. Cold water circulates in the metal jacket and rapidly cools the tube after atomisation, ready for the next sample.

Reproducibility of absorbance peaks is relatively poor with variations of 10% not uncommon. However, more consistent results can be obtained as operators develop a suitable 'technique' for introducing the pipetted sample. Automatic pipetters can further reduce variability to less than 4%.

2.6. Anodic Stripping Voltammetry

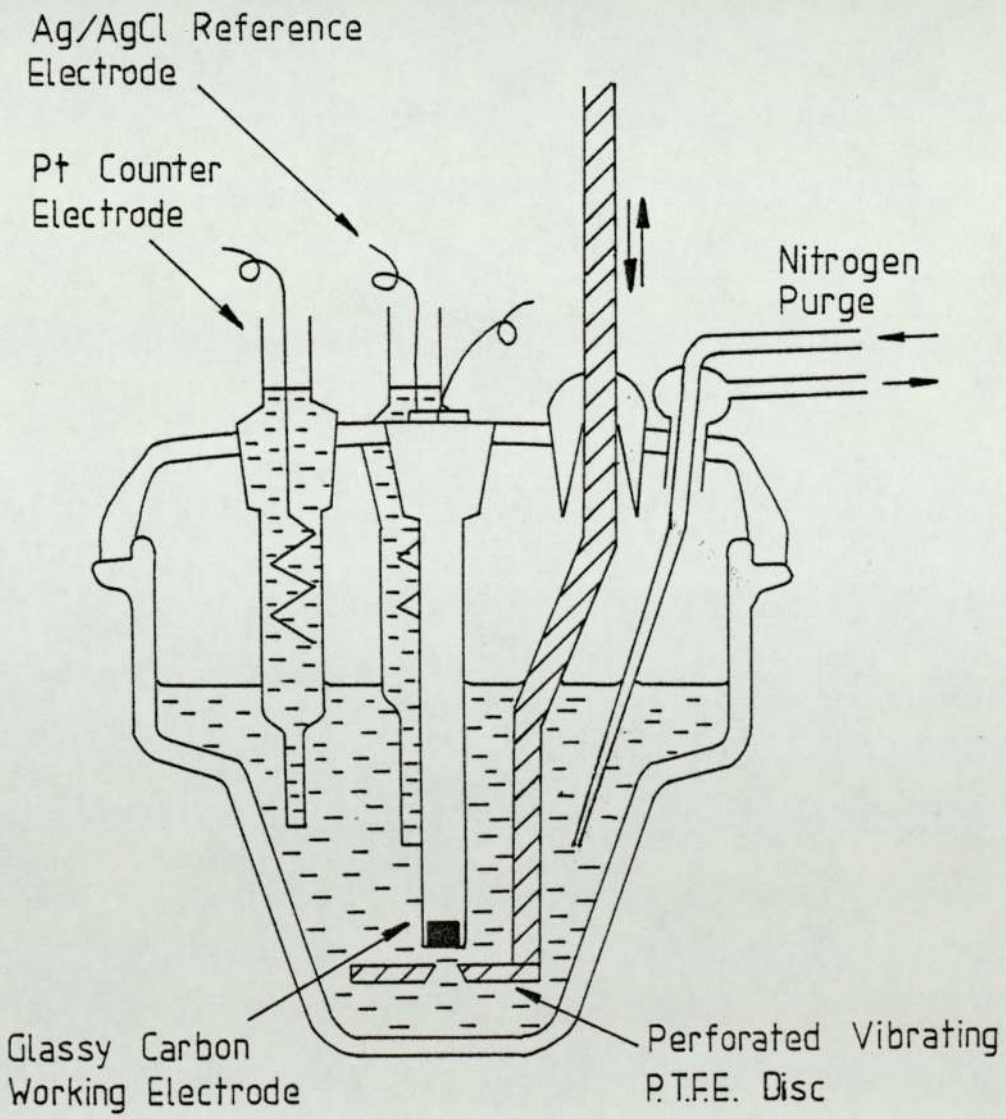
ASV is a very powerful technique, as pretreatment of water samples is most often not required, but sensitivity and specificity are still very high. It also has the advantage of determining both trialkyl and dialkyl lead in a single sample, saving time and reducing inaccuracies. In this investigation triethyl, diethyl, trimethyl and dimethyl lead ions were analysed by this method.

Stripping Voltammetry differs somewhat from the more commonly used mercury dropping electrode devices. Detailed descriptions can be found in most modern polarographic texts [60], but only the relevant ASV technique shall be discussed here.

The procedure consists of two main stages. There is an initial deposition of the metal ions, from solution, onto a working glassy-carbon, mercury-film electrode held at a certain critical potential. Mercury (as $HgCl$) is added to the electrolyte to facilitate this deposition.

Subsequently there is the stripping of the metal back into solution during a controlled change in potential. During this oxidation of the metal, the current increases rapidly and peaks at the critical potential. This peak is proportional to the concentration of metal ions in solution. Thus, the electrochemical reduction of the metal ions, (first stage), must be a reversible process otherwise stripping will not occur. The voltammetry cell used for all analyses is shown in Fig. 2.4.

FIG 2.4 ANODIC STRIPPING VOLTAMMETRY CELL



Qualitative deposition of the analyte ions is achieved during a predetermined time interval, and at a specific reduction potential, so that ideally, only the analyte ions are deposited[61]. By reducing one metal ion species only, the complex interactions of amalgams are avoided during stripping. This allows current peak heights to be recorded and not peak areas, thus increasing sensitivity.

Dialkyllead ions are preferentially deposited at a potential of -0.8 V, and at -1.0 V to -1.1 V both trialkyl and dialkyl lead are deposited[61]. The trialkyllead concentration is therefore calculated by subtracting the dialkyllead concentration obtained from the first deposition at -0.8 V. The solution is stirred continuously during this process to avoid erratic fluctuations of the current that might occur due to natural convection of the solution, if it were not mixed. Stirring was by a PTFE vibrating disc (50Hz), perforated with a central conical hole.

The quantity of metal deposited is proportional to the concentration of the metal's ions in solution, the depostion period and the potential applied. On reversing the potential, the metal is stripped back into solution in its original form, when the critical oxidation potential is reached. The critical potentials for dialkyl and trialkyl lead ions are almost identical, suggesting that the net electrode reaction is the same for all species. The oxidation potential of metallic lead to Pb^{++} is also similar, and it is this latter oxidation process that is occurring in all cases. By recording the current peak height generated at the oxidation potential, and comparing this to a similar peak from a standardised addition of an alkyllead standard, usually trimethyl or triethyl, the concentration can be accurately determined.

The presence of free inorganic lead would interfere withionic alkyllead determination. This interference is prevented by addition of EDTA or

CDTA, both of which form electrochemically inactive complexes with inorganic lead, under suitable pH conditions[61].

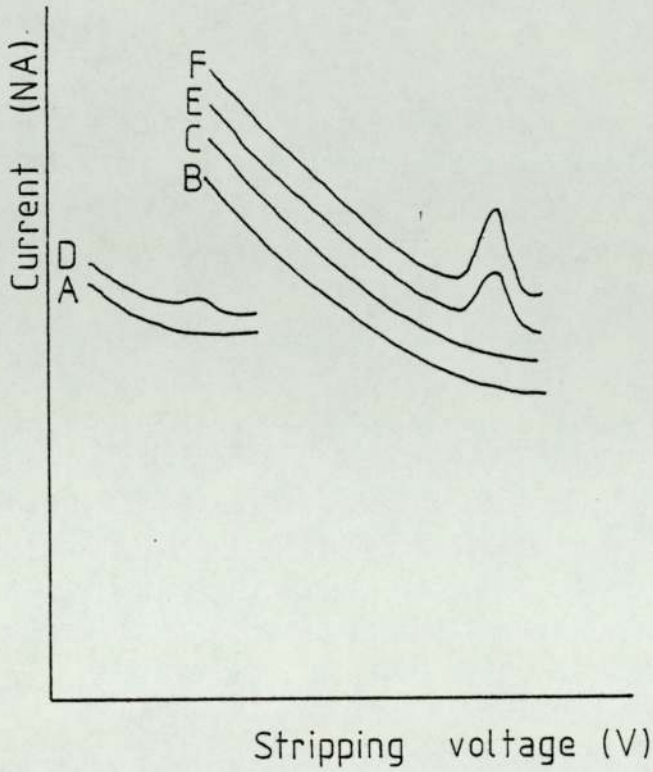
The cell electrolyte is buffered to pH 5 and contains sufficient CDTA to complex the inorganic lead present in the sample and reagents. A 30% sodium chloride solution is also added to minimise the formation of dimethyllead, from a reaction between mercuric mercury and any trimethyllead in solution. The corresponding triethyllead reaction does not occur[61]. Typical recorded current peaks for trialkyllead determinations are shown in Fig. 2.5.

2.7. GLC Mass Spectroscopy

In all of the MS analyses undertaken in this investigation, a gas-liquid chromatography column was used to separate any TAL present, if any, in the sample prior to mass spectrometric determination. Only TAL compounds were quantified by this method. The columns are used to separate volatile substances in time, (in this case TAL), each compound having a unique retention time within the column. Thus, if a known compound has the same retention time as that of an unknown molecule, it is likely that they are the same. A different column is used to confirm this.

Two types of column were employed, the choice of which depends upon the solvent containing the TAL. For biological extractions where toluene was the solvent, a 6 ft packed column of 5% carbowax 20 M packing was used. For aqueous extractions where hexane was used as the solvent, a 50 meter capillary column (internal diameter 0.03 mm) containing OV1 was incorporated into the system. In both cases the carrier gas was helium, regulated at 20 ml min^{-1}. In general, capillary columns having a shorter retention time, allow for higher recorded peaks at the ion detector within the mass spectrometer and are therefore more sensitive. However, for toluene containing samples the other column was found to be

FIG 2.5 TYPICAL RECORDED CURRENT PEAKS FROM A.S.V. R_3Pb^+ AND R_2Pb^{++} DETERMINATIONS



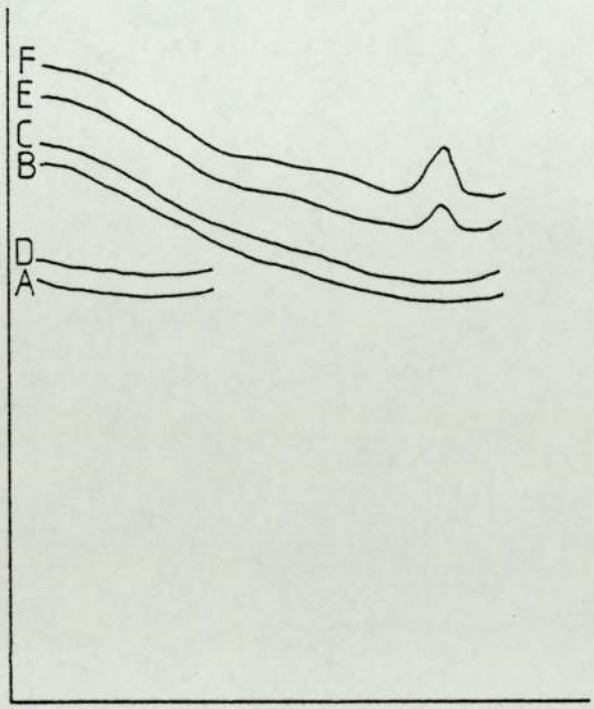
A R_2Pb^{++} blank

B C R_3Pb^+ blanks

D R_2Pb^{++} sample

E R_3Pb^+ sample

F sample + standard



more suitable[62].

Once the various lead compounds have been separated by the column, they enter the mass spectrophotometer. The instrument detects specific ion masses generated under vacuum, from electron bombardment of analyte molecules. Electrons emitted from a heated filament pass through slits and enter an ionization chamber as a finely collimated beam. Positive ions are formed when the high energy electrons knock out orbital electrons of the analyte molecules. These ions are initially repelled and then accelerated by a large potential difference, towards the mass ionization detector. The ions pass through a filter system which consists of a series of horizontal rods having DC and radiofrequency currents applied to them in different planes. The combined effects of these forces cause the ions to spiral down the length of the rods until they reach the photomultiplier.By altering the currents, specific ion masses can be separated from all other masses which collide with the rods before reaching the detector.

Ions striking the photomultiplier initiate a multiplying electron current which is amplified and converted into a suitable readout. For quantitative work, only specific ion masses of the fragmented parent molecule are scanned to increase sensitivity, as the detector has more time to focus on just one or two masses. However, a whole mass spectrum can be recorded for qualitative identification of compounds.Such spectra of TML and TEL can be seen in Figs. 2.6. and 2.7.

As the parent molecule fragments under electron bombardment, formation of certain ion masses is more probable than others. Both TML and TEL produce several ion fragments of similar mass that always show the same ratio of abundance. For example, a triplet at 251, 252 and 253m/e always occurs for TML, with ratio of 1:1:2 (Fig. 2.6.). A similar triplet

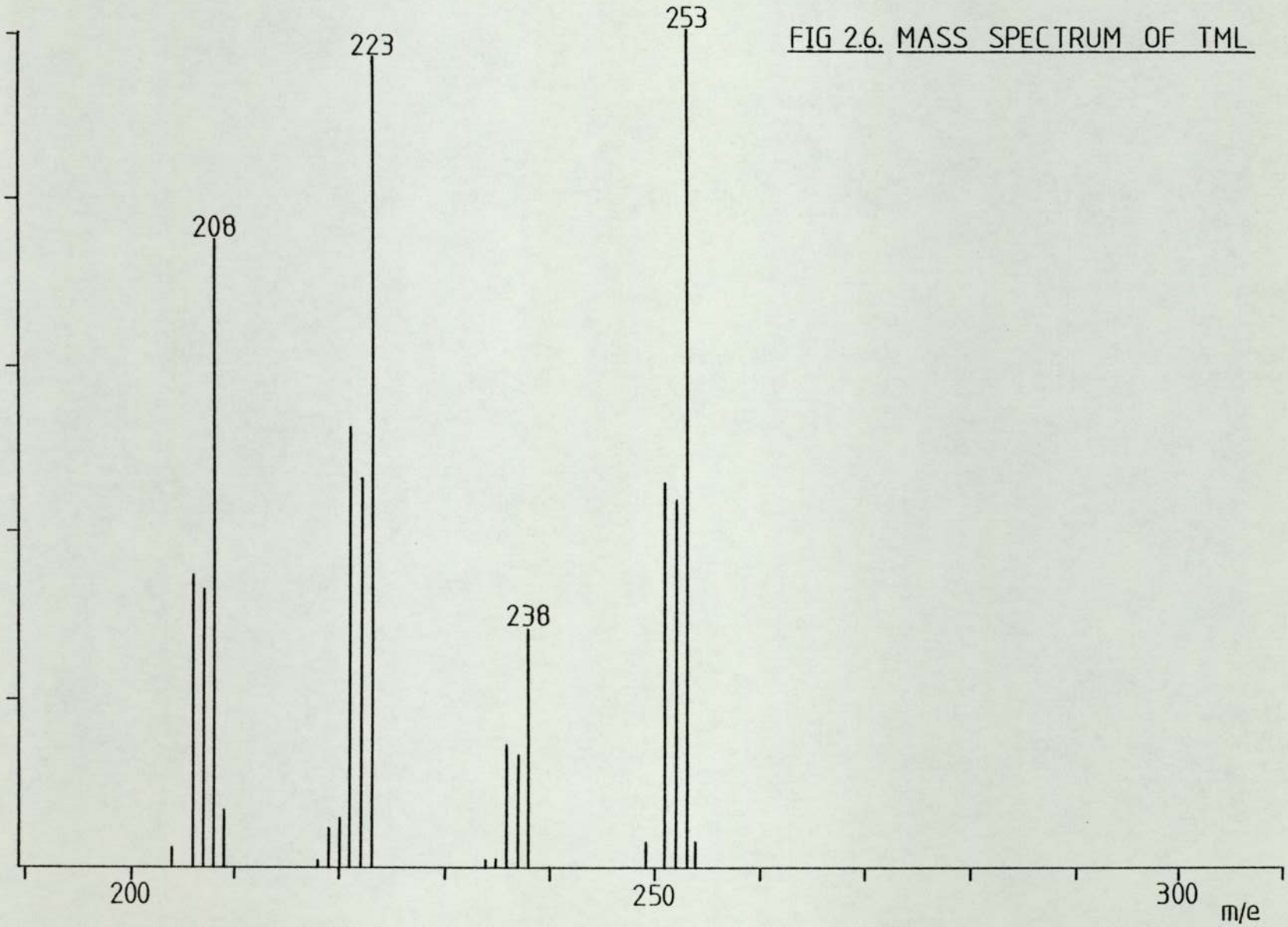
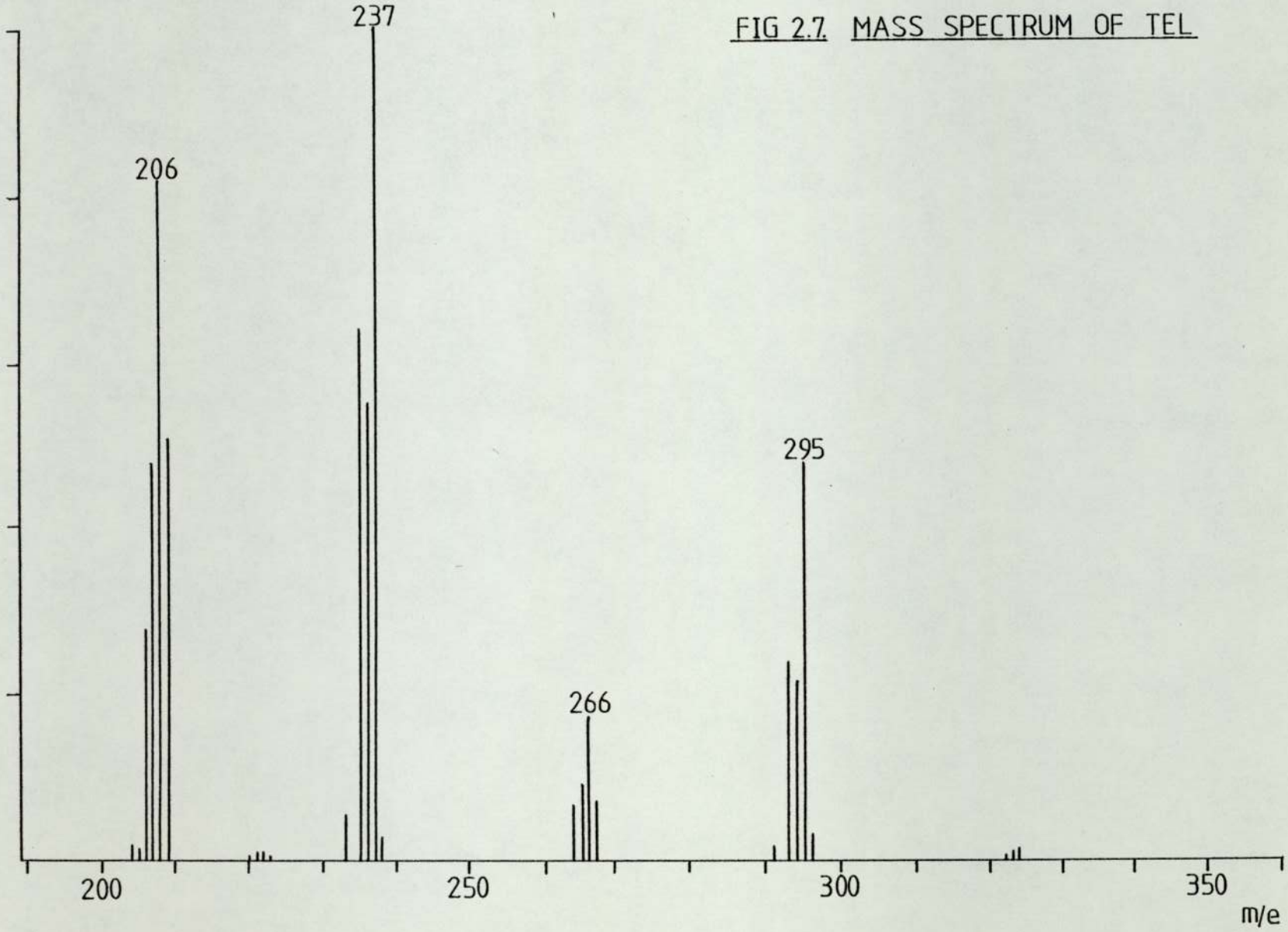


FIG 2.6. MASS SPECTRUM OF TML

FIG 2.7. MASS SPECTRUM OF TEL



for TEL, with the same ratio can be seen at 293, 294 and 295 m/e (Fig.2.7). By focusing on these mass groupings and with the knowledge of the retention time of analyte molecules in the column, very specific quantitative determinations can be made.

2.8. Analysis of the Photosynthetic Activity of Phaeodactylum tricornutum

Experiments conducted to determine the effects of alkyllead compounds on the marine diatom Phaeodactylum tricornutum exploited the algal photosynthetic uptake of radioactive $^{14}CO_3$ in solution.

The technique used is similar to that of Maddock and Taylor[51], based on the measurement of organic productivity in the sea method by Steeman-Nielson[63]. A standardised quantity of the algae is exposed to a range of alkyllead concentrations and incubated under standard conditions for 6 hours with a known amount of labelled carbonate (added as $Na_2{}^{14}CO_3$). At the end of the incubation, the amount of ^{14}C uptake is determined by liquid scintillation counting (LSC) and, after correction for non-photosynthetic uptake, the photosynthetic activity can be calculated as a percentage of the 'normal' (control) activity.

There are several advantages in using labelled ^{14}C uptake as opposed to other methods of toxicity estimation. The photosynthetic activity or growth of an alga is an appropriate variable to assess and, as only living cells contribute to the activity, the presence of dead cells is not a problem. The adsorbtion by dead cells is eliminated by subtracting the activity of dark-grown cultures. P.tricornutum cells have silaceous cell walls and therefore dead cells do not rapidly decompose under normal conditions, making live-cell counting a difficult task. Indeed, by measuring the radioactivity of exposed cultures, the time consuming and laborious counting of individual cells is avoided completely. Another advantage is that, an entire batch of experiments can be run

from start to finish in a single day.

The major disadvantage of this technique, is the requirement of stringent safety precautions that run hand in hand with the use of all radioactive materials. ^{14}C is β-particle emitter with a half-life of approximately 5500 years[64], resulting in contaminated areas remaining active for substantial periods. Extra precautions are needed to contain the exposed cultures because of the nature of the $^{14}CO_3$ which forms $^{14}CO_2$, especially in acidic conditions. All radioactive materials, including exposed cultures, were kept in an efficient fume-cupboard.

2.9. Liquid Scintillation Counting

The general procedure for liquid scintillation counting is relatively simple. After incubation the algal cells are separated from the 'hot' (radioactive) culture medium by membrane filtration. The cells are washed in 'cold' (non-radioactive) medium by flushing it through the filter with a syringe, and then both filter and algae are dissolved in a liquid scintillation cocktail.

The cocktail is comprised of a solvent and a fluor. The solvent should ideally exhibit minimum light absorption, maximum transfer of energy from the radiation source (algae) to the fluor, very high purity to minimise energy transfer loss and high solubility for the radioactive material. The fluors are present to convert energy from the radiation source into scintillations or light photons. They should therefore have a high energy absorption from the solvent molecules, be soluble in the solvent, be chemically stable and have a short fluorescence decay time[64]. In reality a compromise between these factors is usually achieved.

In the present study, the cellulose nitrate membrane filters and algae were dissolved completely by the cocktail used ('Cocktail T' BDH Ltd.), producing a clear solution prior to counting. When ready for counting the vials containing the samples were placed in the liquid scintillation

counter. However, straightforward counting to estimate the activity was not possible because of quenching.

Quenching is the reduction in sample-emitted light reaching the instruments detector (photocathode). It can be caused by many processes, such as: chemical interference of the solvent to fluor molecule energy transfer, coloured material absorption of fluor-emitted photons or optical interferences due to dirty glass components in the light path.

Obviously there is likely to be a certain amount of quenching in every sample. This problem can be overcome by several methods but in this case external standardisation of the sample was used. A γ-emitting source is placed alongside the sample inside the counting chamber, thus generating a degree of fluorescence in the sample. The amount of quenching present influences the fluorescence which is counted and compared to the counts from the sample only. The resulting ratio is compared to calibration charts allowing the counting efficiency to be computed. Once this efficiency is known, compensation for quenching can be achieved. Once the control cultures have been counted, they are corrected for non-photosynthetic absorption of ^{14}C. Control cultures are exposed to the same controlled conditions as all other cultures, but do not contain the test contaminant. These controls give an indication of the 'natural' photosynthesis of the algae, and are taken as the maximum (100%) activity. Cultures exposed to the test substance are assessed as a percentage of the control activity. Thus, the greater the toxicity, the lower the percentage photosynthetic activity.

CHAPTER THREE

EFFECTS OF ALKYLLEAD COMPOUNDS ON MACOMA BALTHICA AND SCROBICULARIA PLANA

EFFECTS OF ALKYLLEAD COMPOUNDS ON MACOMA BALTHICA AND SCROBICULARIA PLANA

3.1. Introduction

British antiknock fluid (80%) is exported mainly by sea[65]. Consequently environmental contamination from a major spill is most likely to occur in coastal or busy shipping lane areas. Both TEL and TML have densities greater than that of water (1.650 gml^{-1} and 1.995 gml^{-1} respectively[17]) and would rapidly sink to the sea bed. Sedentary animals such as sediment dwelling bivalve molluscs or polychaete worms in the immediate vacinity of such a spill would then receive the greatest exposure. Similar animals inhabiting areas in the path of any dispersion plane would also be directly exposed to lead alkyls. Because of the highly insoluble nature of TAL, if the prevailing currents were relatively slow, these organisms would be the only ones subjected to TAL. However, dispersion of lead alkyls from pools located on the sea bed depends predominantly upon the current and turbulence around the pool, each location having its own unique profile[66]. Molecular diffusion under these conditions would be a negligible contribution to dispersion.

In the only major spill of antiknock compound that has been studied in any detail, the 'Cavtat' incident, it was estimated that contamination from the pool on the sediment would only have extended 500 metres at worst, and most lead alkyls would remain within a much smaller area[66]. Decomposition of the TAL compounds would produce the lower analogue forms, the amounts of which would be dependant partly upon the degree of illumination and therefore the depth. These ionic species are much more soluble in seawater and would presumably disperse further than TAL, although dilution and dispersion would prevent large localised concentrations.

This probable behaviour of antiknock compound spilt at sea makes the study of the relationships between bottom-dwelling organisms and

alkyllead most pertinent. Bivalve molluscs are well known for their ability to accumulate heavy metals from seawater[67-73]. This phenomenon is very important when considering the large variety of mollusc consuming creatures, including man. It is plausible that the alkyllead compounds could be passed along food chains, ultimately ending with man, if a spill occurred in heavily fished areas.

There have been several reports of heavy metal toxicity to man from shellfish consumption[75-77], the most infamous being the Minamata Bay disaster[78]. More recently it was reported that alkyllead poisoning may have been responsible for the deaths of large numbers of birds on the Mersey estuary in 1979[52]. The birds, mainly dunlin, are thought to have picked up the lead through their diet of mainly bivalve molluscs, which may have accumulated alkyllead from sources within the estuary. Both incidents are discussed in more detail in Chapter 1.

To generate more information about the toxicity and accumulation of alkylleads in bivalves relating to the afore mentioned Mersey estuary incident, two species were exposed to various organolead compounds. By determination of the toxic concentration to these animals, and from measurements of alkyllead accumulation within them, it was thought it might be easier to understand how the incident occurred.

3.2. Animals Used

Two species of bivalve commonly occurring in most British estuaries were chosen for this investigation. Both Macoma balthica and Scrobicularia plana are relevant to the 1979 Mersey estuary incident as they are potential food sources for coastal birds. Indeed Macoma constitute a large proportion of the diet of many waders on the Mersey itself.

The animals have been classified as follows[79]:

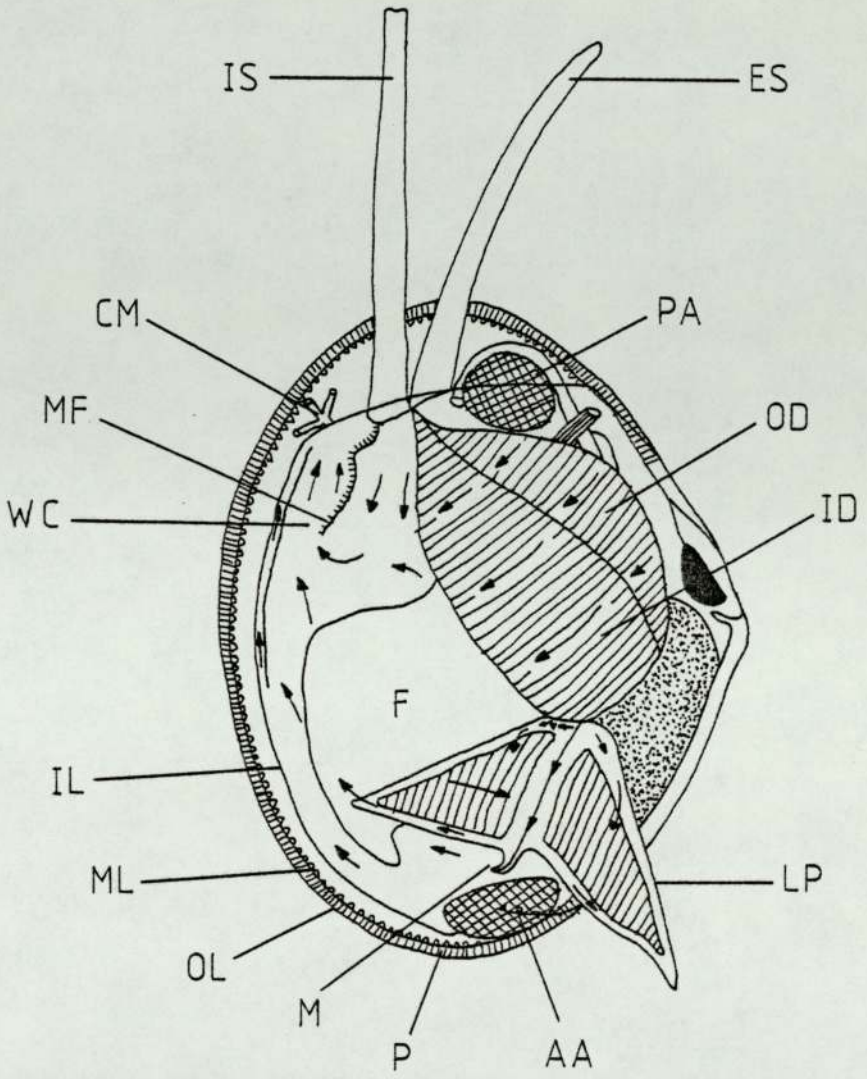
Class	Bivalvia	
Subclass	Lamellibranchia	
Order	Heterodonta	
Superfamily	Tellinacea	
Family	Tellinidae	Semelidae
Genus	Macoma	Scrobicularia
Species	balthica (L.)	plana. (Da Costa)

As would be expected from their membership of the Tellinacea, both species show similar adaptive structures to a shallow burrowing and filter-feeding habit. These similarities can be seen in Figs 3.1. and 3.2.

Both species are mud-dwelling, filter feeders using their ciliated demibranchs to sort particles collected by the inhalent siphon. The animals are predominantly deposit feeders, in that the muscular, extensible inhalent siphon extends some way out of the burrow and actively sweeps to and fro, sucking up sediment. However, when the tide has covered them, both species can suspension feed, by filtering the phytoplankton from the seawater. Scrobicularia burrows 5.2 cm. into the substrate, maintaining contact with the more aerobic surface waters with its siphons (Fig.3.3). Macoma has a similar habit, but remains closer to the sediment surface. A more detailed description of the biology of both species is available in the literature[80-82].

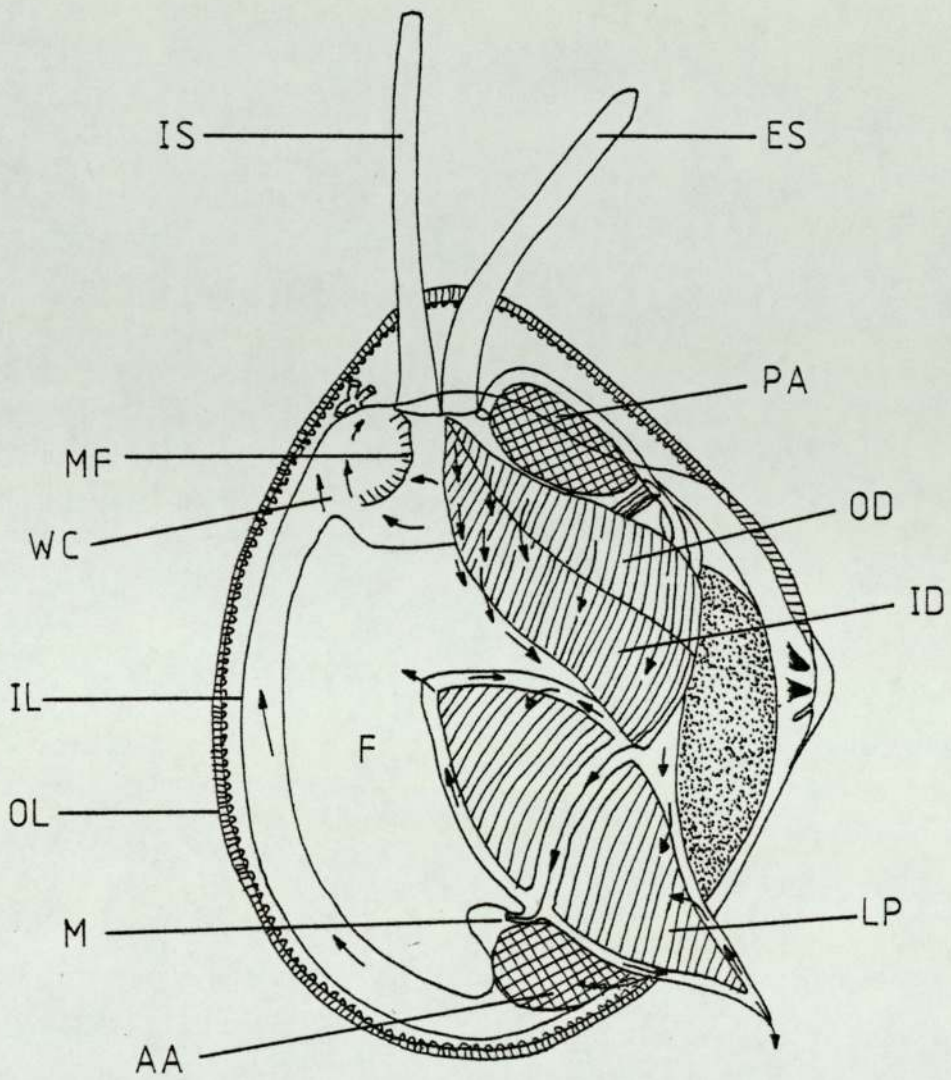
All animal samples were collected from either of two sites on the Dee estuary, at Thurstaston (Cheshire) and Mostyn (N.Wales). Approximate Ordinance Survey co-ordinates are 835 225 and 825 155 respectively, (O.S. Map No. 108). This estuary was chosen as the sampling site for several reasons. It supports dense populations of Macoma and Scrobicularia, unlike the Mersey estuary which has very few Scrobicularia[52]. The estuary is within a convenient travelling time and distance from the Aston laboratory, so that the animals spent the minimum time in transit.

FIG 3.1
SCROBICULARIA PLANA (DA COSTA) x2·5



IS	Inhalent Siphon	M	Mouth
CM	Cruciform Muscle	P	Periostracum
MF	Mantle Fold	AA	Anterior Adductor
WC	Waste Channel	LP	Labial Palp
IL	Mantle Edge Inner Lobe	ID	Inner Demibranch
ML	" " Middle Lobe	OD	Outer Demibranch
OL	" " Outer Lobe	PA	Posterior Adductor
F	Foot	ES	Exhalent Siphon

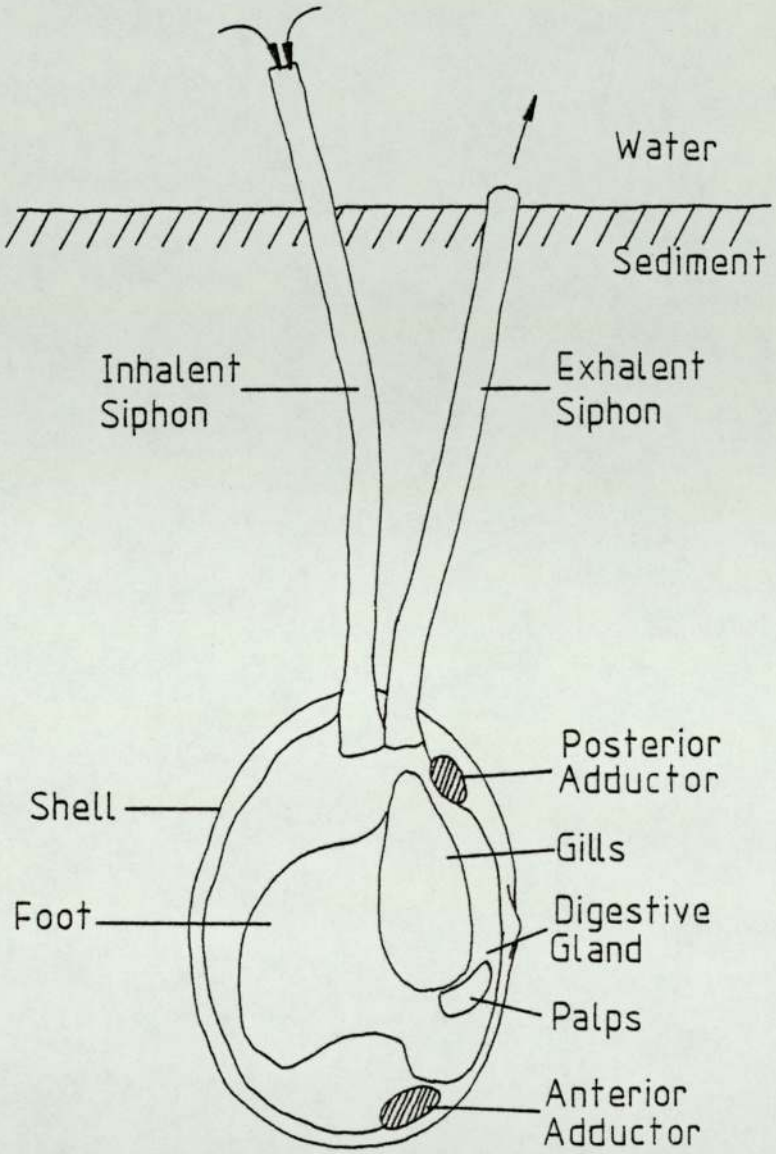
FIG 3.2
MACOMA BALTHICA (L) x5



IS Inhalent Siphon
MF Mantle Fold
WC Waste Canal
IL Mantle Edge Inner Lobe
OL " " Outer Lobe
M Mouth
F Foot

AA Anterior Adductor
LP Labial Palp
ID Inner Demibranch
OD Outer Demibranch
PA Poster Adductor
ES Exhalent Siphon
/ Ciliary Current

FIG 3.3
NATURAL POSITION OF SCROBICULARIA



The estuary itself and the bivalves within it, contain negligible amounts of organolead[52,57], although inorganic lead levels are not particularly low[52]. From the present study, analyses of both species taken from the estuary, suggested alkyllead levels below the detection limit of 0.02 mg l^{-1}.

The molluscs were collected by hand at mid-tide level, during an ebb tide, and were immediately transferred to polythene bags containing estuary water. Several bags were used to prevent any damage to the animals during transit to the laboratory. The time taken from collection to arrival to the laboratory was usually about 4 hours. The specimens were quickly cleaned of excess mud and placed in 35 l stock tanks containing aerated, artificial seawater (34‰), kept at a constant temperature (10°C ± 1°C). Overcrowding of the stock tanks was avoided to maintain the bivalves at a maximal condition prior to their use in experiments. Illumination was by subdued, natural daylight.

The animals were collected between May and July of 1981, 1982 and 1983. Although there may have been some variation in animals from the different years, they should have been of similar general condition, with respect to gonadal development and condition factor (see below).

Scrobicularia is more tolerant to low salinities than Macoma, and can therefore penetrate further towards the source of the estuary[68]. However, the animals collected in this study were taken from areas near to the mouth of the estuary, where average salinities would be relatively high, and at high tide would expose them to coastal seawater. Thus it was acceptable to maintain both species in full strength seawater in the laboratory. This had several advantages when considering comparisons with other sudies of alkyllead effects on marine organisms, as these also used 100% seawater. By using seawater of a similar salinity to that used in other studies, the availability of the various compounds to the

animals, would be the same as that to other organisms involved in these studies.

3.3. Bioassay Methods

Toxicity testing requires detailed consideration of the biology of the animal under investigation, as well as the experimental conditions, before any exposure may begin. There are a great number of possible variable factors in the experimental environment which may influence the degree of toxicity of a particular substance. These will be reflected to a large extent, in the animals' behaviour and metabolism, before and during exposure to the substance being tested. Although bivalves, and especially estuarine species, are relatively tolerant to environmental variability, they are bound to experience some stress when collected from their natural habitat and placed under laboratory conditions. These conditions, and other variables affecting bivalve performance, are briefly discussed below.

Seasonal variation in the animals' total body weight must be considered in any toxicity/accumulation study. Fluctuations in weight are influenced mainly by the reproductive cycle and the diet or food supply. Generally gonadal development in British bivalves occurs in autumn and winter, with spawning taking place in spring. Immediately after spawning, the bivalve is at its minimum body weight. Maximum weight occurs in summer, after advantage has been taken of the abundant phytoplankton food supplies. As food availability increases in the spring, so the metabolic rate of the bivalve increases, which in turn will affect the rate of accumulation[83]. The type of food utilised by the animal is important, especially when monocultures are used in laboratory tests[84]. Obviously some food sources will be more easily assimilated, or will yield higher calorific values per unit consumed, than others.

The effect of animal size upon the metal content of the shellfish makes it important to use similarly sized animals in accumulation experiments. Generally metal content is linearly related to the log of body weight, and smaller individuals contain higher concentrations than larger ones, when exposed to the same conditions[85,86]. Rates of uptake and loss of lead in the common mussel (Mytilus edulis) have been shown to be less in larger animals than in smaller ones[67,71].

Salinity influences metal content in bivalves, this being particularly relevant to estuarine species. Fluctuating salinity regimes applied to Mytilus edulis influenced metal uptake from solution in a complex manner [87-89] partly due to a valve closure response exhibited by the animals when exposed to large, rapid salinity changes. Stable seawater salinities have a lower effect on metal uptake[87], although one study [90], has shown a decreased uptake with steadily increasing salinity.

Temperature changes alter the respiratory metabolism of bivalves, including Macoma[91]. Consequently, if the experimental temperature differs from that of the animals' natural temperature cycle, alterations in metabolism should be expected[92].

Littoral or intertidal bivalves, subject to cyclic emersion and immersion by the tides, regulate their metabolism and behaviour accordingly. For example, in experiments with Mytilus edulis, cadmium uptake was significantly affected by alterations in emersion/immersion periods[93]. Animals which were periodically 'drained', pumped water proportionally longer when immersed, than animals continually under water, although the latter group accumulated the highest cadmium concentrations overall.

These factors exemplify the importance of using the optimal test conditions for the specific animals under investigation. Only after careful consideration of all the major variables should the bioassay

system be chosen.

In the present study the test system for trialkyllead exposure consisted of 2 l, open-topped polycarbonate tanks which were covered with glass plates to prevent cross-contamination from the spray generated by aeration. Each tank was aerated with laboratory air, bubbled through the seawater by standard aquarium pumps (Rena 301). Oxygen saturation was greater than 95%. The tanks were placed in a large, refrigerated cabinet, which maintained the temperature thermostatically at 10°C ± 1°C.

The system for TEL was similar to that for the trialkylleads, but with some important differences. TEL has a higher volatility and is only sparingly soluble in seawater. Aeration would have increased the rate of loss of TEL from the system. To maintain the safety precautions, aeration was therefore excluded, in spite of the probable deleterious biological consequences. The test tanks were smaller (1.4 l), made of pyrex and were painted black on the outer surface. The smaller volume was to confine the area from which TEL could be lost from the system. The black paint was to minimise photolytic decomposition. Glass plates were placed over the tanks, leaving minimal air space, thereby also reducing the area of TEL loss, and preventing TEL escaping from the seawater into any gaseous phase.

Artificial seawater[94] (34‰) was used in all the experiments, because of the problem of obtaining large stocks of natural seawater. The pH was 7.9 ± 0.2. Generally the seawater was changed regularly, with the exception of the 72 µl l^{-1} TEL experiments which shall be discussed later. Immediately after the change, the fresh seawater was dosed with the relevant compound to achieve the required alkyllead concentration. The concentrations were monitored from the initiation of an experiment, right through to the end, every few days when possible. This was done

because some difficulty was encountered in maintaining a steady concentration throughout the experiments.

A flow-through system was not possible with the volumes of artificial seawater available, so a static system was adopted, with regular and frequent seawater changes. For all concentrations of alkyllead investigated a series of replicate tanks were employed, as were control tanks, for each compound under investigation.

The animals used in each experiment were carefully cleaned of all sediment, by gently washing the shells with seawater. They were then acclimatised to the test tanks, which contained 2 l. of artificial seawater, for a minimum of 7 days, before an experiment was started. Animals of a similar size were used. The mean length of Macoma was 15.5 mm $\pm$ 1.04 mm (range 14-17 mm), and that of Scrobicularia was 40.4 mm $\pm$ 1.57 (range 38-43 mm). Because of the greater size of Scrobicularia, fewer of these animals were placed in each tank. The numbers of each species was reduced in TEL tests, because of the smaller seawater volumes. Only one tank for each concentration of Me_3Pb^+ was used for Macoma because of the limited numbers of animals.

Scrobicularia appeared to show no sign of distress prior to alkyllead addition, lying horizontally on the floor of the tanks. However, Macoma were not observed to feed under the same conditions. Several artificial substrates were tried in an attempt to overcome this problem. A matrix of glass helices of varying diameter (3 mm - 10 mm), proved the most successful substrate, with the animals burrowing soon after being placed on the surface. The helices were added to a depth of approximately 2 cm, sufficient to enable the animals to attain a vertical orientation. Natural sediment was excluded from the test system because of its complex chemical and physical nature. It would be probable that the alkyllead compounds in solution above the sediment would be chelated, adsorbed or

complexed in some other way with components of the sediment, causing a rapid decrease in concentration. This effect is clearly demonstrated in Fig.3.4.

An initial seawater concentration of 1.0 mg l^{-1} Me_3Pb^+ was made up above a 4 cm layer of untreated Dee estuary sediment. The concentration fell rapidly, the half life being approximately 35 hours. A system containing sediment would, therefore, make steady, controlled dosing of animals extremely difficult. This is apart from the additional problems of deoxygenating bacteria and the deposit or filter-feeding behaviour of the bivalves further complicating dosage calculations.

Both species were fed on a single species of unicellular diatom, Phaeodactylum tricornutum, which was cultured in the laboratory. Each tank was dosed with 100 ml of a dense culture, every few days. The small dilution involved in adding this volume was compensated for by adding the required amount of alkyllead stock to maintain the concentration.

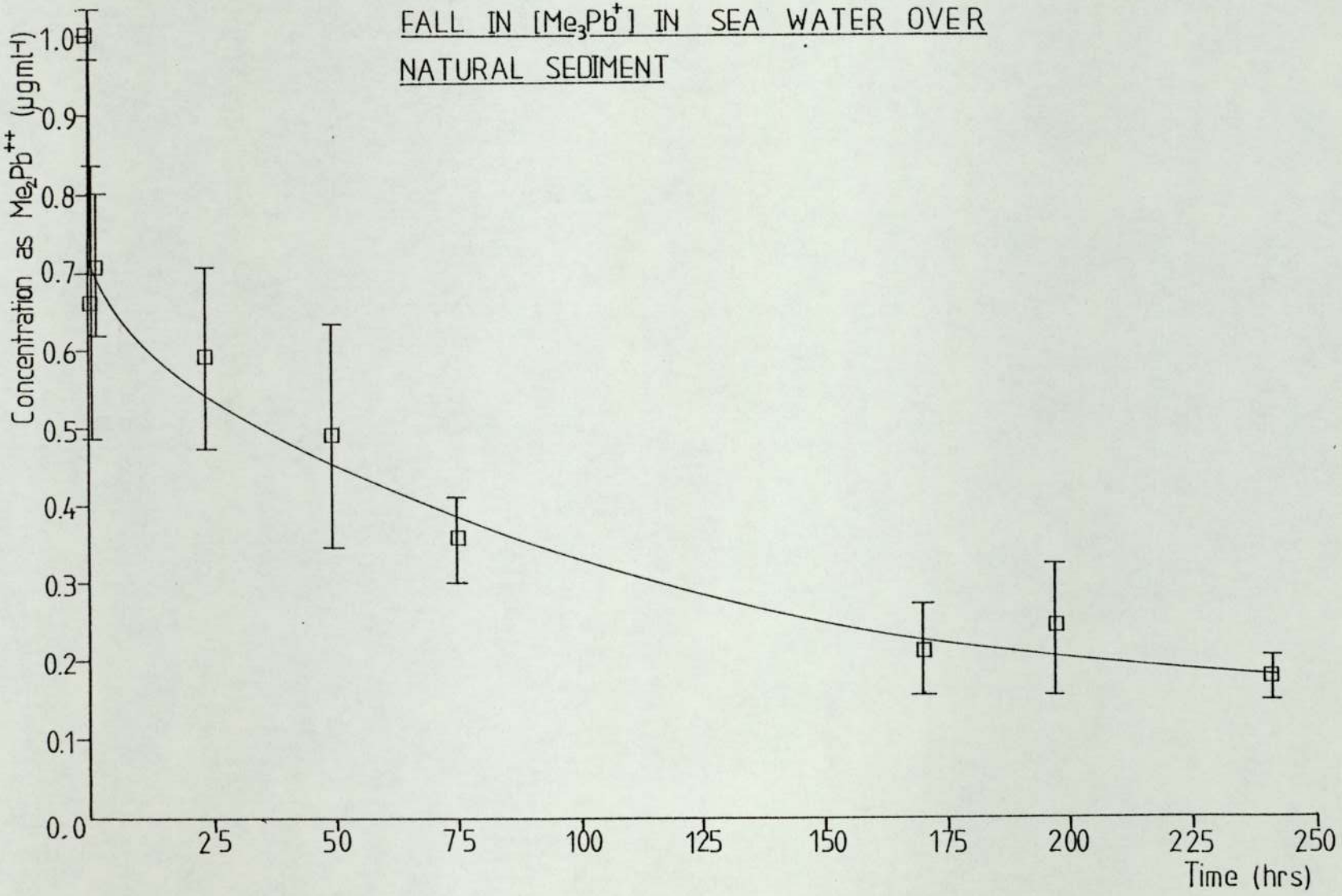
During the experimental runs, animal observations were made daily, to to record the number of mortalities. Dead animals were removed immediately death being determined when a gaping individual failed to respond to tactile stimulation to the soft tissue. All exposed animals were handled gently, using forceps.

3.3.1. Alkyllead Compounds Used

Analyses of captured or dead birds and shellfish on the Mersey estuary in 1979 revealed that substantial percentages of the total lead present were trialkyllead[52]. Much lower proportions were found to be TAL and even less to be dialkyllead. It seemed relevant, with respect to these findings, to primarily assess the effects of the trialkyllead compounds and TAL. The limited research time, and the likely lesser importance of dialkyllead effects, determined that these compounds would not be

FIG 3.4

FALL IN $[Me_3Pb^+]$ IN SEA WATER OVER NATURAL SEDIMENT



investigated under the bivalve toxicity procedure.

The compounds investigated were, therefore, Me_3PbCl, Et_3PbCl and TEL. TML was not used because of the difficulty in obtaining sufficient pure quantities (TML is highly unstable in its pure form), and the extra risks involved in using this material in open systems. TML has a much greater volatility than TEL, and is therefore more dangerous to the handler. All alkyllead compounds were donated by the Associated Octel Co.Ltd. Both trialkyllead salts were as the chlorides, and all materials had purities of >98%.

For the purposes of dosing the test tanks, stock solutions of the trialkyllead chlorides were made in distilled water to 100 mg l^{-1} or 1000 mg l^{-1} and stored in a refrigerator at 4°C. The high concentrations enabled small additions to be made to the seawater, thereby avoiding significant salinity changes. TEL is very insoluble in water, so that small droplets of neat compound were added instead. This procedure also minimised the contamination from triethyllead, formed by TEL decomposition when stored. The TEL was washed prior to addition with a dilute ammonia solution to remove any decomposition products.

3.3.2. Toxicity Test Design

Experiments aimed at determining the toxicity of materials in aquatic systems usually follow the many commonly used and acknowledged protocols available. The advantage in following one or more of these procedures obviously lies in the ease with which comparisons can be made with other studies implementing the same techniques. It is useful to be able to determine quickly the relative toxicities of different materials, without having to conduct time consuming experiments. Many tests for acute lethality involve short term exposure, as in the 96 hour LD_{50} or LC_{50} determinations. However, short term toxicity tests are not always

convenient. There are a small number of animals that are capable of exhibiting avoidance behaviour when exposed to detrimental conditions. These animals include many molluscs, especially lamellibranchia, which can isolate themselves from their external environment.

In the present study, both Macoma and Scrobicularia are capable of such behaviour by means of valve adduction. Once the shell is completely closed, the animal resorts to anaerobic respiration and bradcardia (slowing the heart beat). The length of time that the animals can remain in this condition may be considerable. For example, Scrobicularia has been shown to remain in isolation for 5-7 days, when exposed to various salinity regimes[95]. As this period is longer than the 96 hours usually applied to LD_{50} or LC_{50} determinations, such methods with this animal are of little use. Clearly, if acute toxicity estimates are to be made, longer experiments are required.

Before the experiments were run, there was no indication as to how long the animals would survive under exposed conditions, or even if they would use valve closure to avoid alkyllead doses. It was decided therefore, to record the time taken for the animals to die, using a constant dose regime. This enabled comparisons between test compounds of similar concentrations.A convenient measure of toxicity, in this respect, was the time taken for 50% of the test population to die, Lt_{50}.

A study of acute trialkyl and tetraalkyl lead to the common mussel (Mytilus edulis), suggested 96 hour LC_{50} values in the range 0.1 to 1.1 mg l^{-1} [51]. As this work was most closely related to the research in the present study, and because Mytilus can also exhibit valve closure under stress[96], a close regime of similar concentrations was devised. Macoma and Scrobicularia were exposed to triethyl and trimethyl lead chloride at concentrations between 0.05 and 1.0 mg l^{-1}. These values are also probably realistic to concentrations present in the Mersey

estuary, which are most likely to be less than 1.0 mg l^{-1} (62).

It should be noted that the actual concentrations in dosed tanks, analysed during the experiments, varied significantly from those expected (see below). These variations continued throughout the entire series of experiments, making it necessary to monitor the concentrations at frequent intervals.

3.4. Condition Factors

When subjecting organisms to long term toxicity studies, it is important to know their condition, as influenced by the test environment. To establish whether the experimental environment itself caused excessive stress in animals, a quantitive measure of the general condition of the individuals is required. Obviously, if the pre-alkyllead exposed environment causes a large decline in the condition of the bivalves, it would be difficult to conclude that any mortalities were the result of alkyllead toxicity alone.

Many condition factors may be used, each one quantifying a particular parameter of the animal, such as behaviour or biochemistry. An index of bivalve condition developed by Ansell et al (97), relies upon simple measurements of weight, to provide a guide to the general metabolic state of an individual. This condition factor is calculated from the relationship between the wet flesh weight and the total body weight, including the shell.

$$\text{Condition Factor} = \frac{\text{Wet flesh weight}}{\text{Total body weight}} \times 100$$

An increase in condition factor usually indicates growth of the animal and an improvement in overall condition, whereas a decrease is indicative of excessive stress, and a decline in condition. This easily determined index has been used in several studies involving bivalves

(16,41,43,44), giving reliable indications of the stress induced by the experimental system in unexposed (control) animals.

Condition factors for bivalves vary considerably with season, under natural conditions, as gonadal development and spawning will increase and decrease the index, respectively. However, in an artificial environment where temperature is maintained at a reduced value, spawning may be delayed without loss of condition, for up to 12 months[99]. Temperatures of 10°C and below cause such behaviour in Mytilus edulis[79] and other lamellibranchs[92].

Ratcliffe[101] suggests that spawning in Macoma in the Humber estuary occurs between March and May, with the animal requiring water of 7°C-14°C to stimulate the release of the gametes. It is possible, therefore, that Macoma may have postponed spawning in experiments run in the spring, because of the constant 10°C temperature of the seawater. The rapid decrease in condition associated with the period immediately after spawning, would then not be seen in these experiments. Indeed no evidence of spawning, in either species, was observed in either of the bioassays, although most of these were performed after the breeding season. Because of the variation in the exact time of spawning from year to year, most of the toxicity experiments were conducted with animals collected between May and July. By using specimens from this relatively narrow collection period, it was hoped that their initial condition would be similar, before being transferred to the test system.

Because of the limited number of animals that could be used in a single experiment, measurements of condition factor were usually made only at the beginning and end of each experiment, using control animals. However, a more detailed estimate of how condition varied with time, for Macoma and Scrobicularia, was obtained by regular sampling of a control population subjected to the test conditions (Figs 3.5 and 3.6).

FIG 3.5 CONDITION FACTOR VARIATION OF SCROBICULARIA CONTROLS (±1 S.D.)

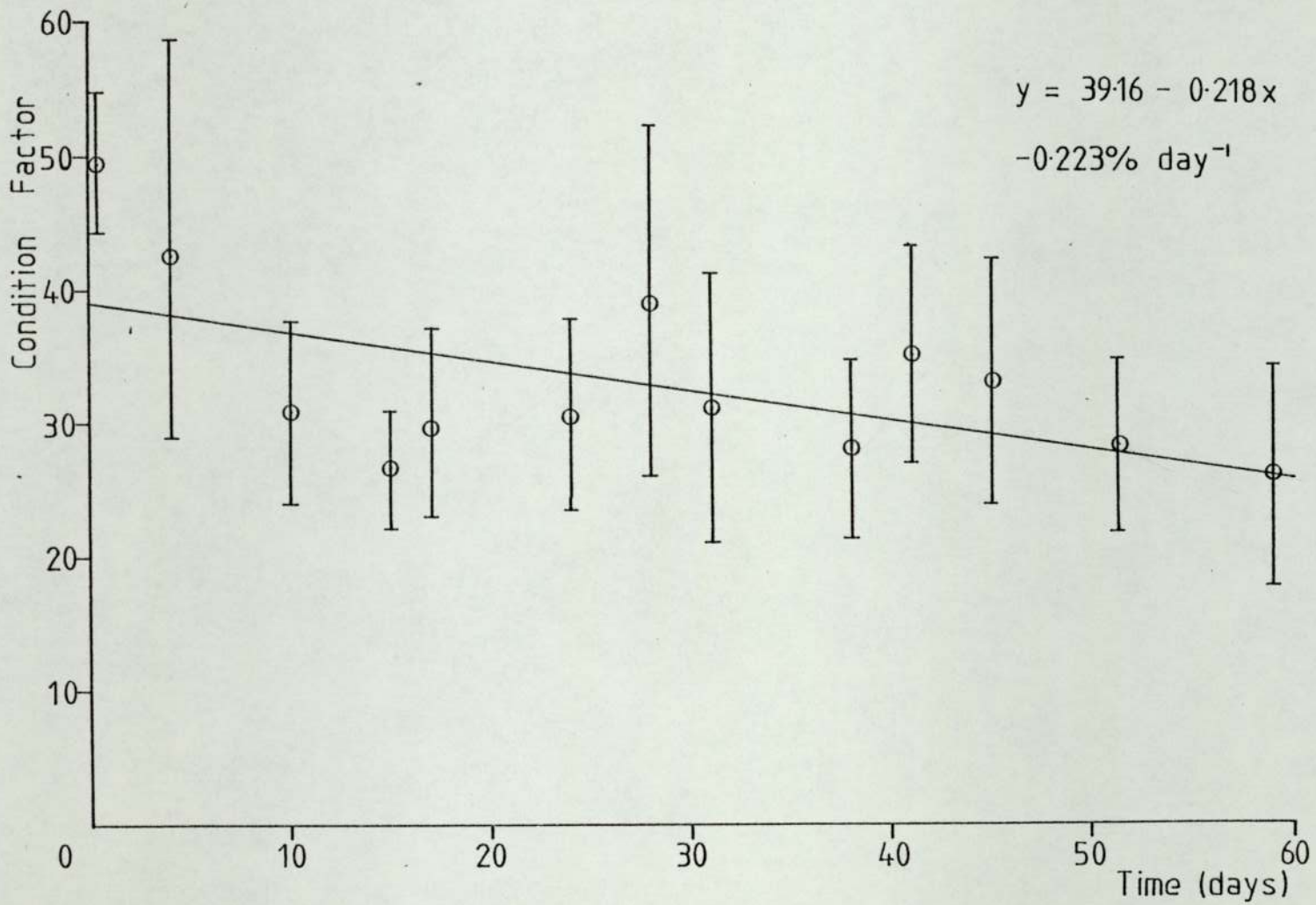
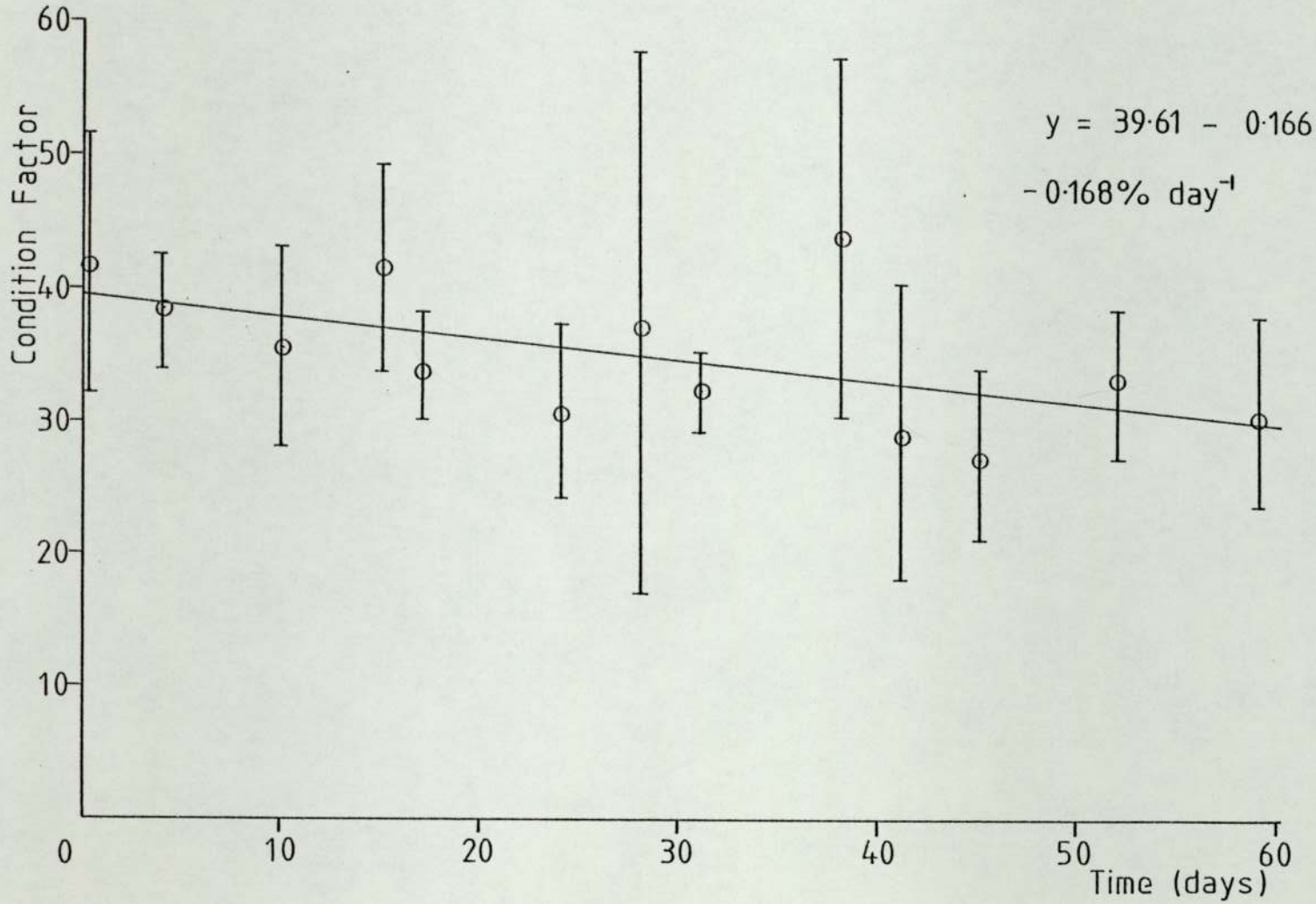


FIG 3.6 CONDITION FACTOR VARIATION OF MACOMA CONTROLS (±1 S.D.)



Animals were removed from the tanks every few days and were immediately opened by slicing through both adductors. The total soft tissue was carefully removed from the shell and excess water absorbed by dabbing with filter paper. Shells were similarly dried. Each individual was then weighed in total and without the shell.

The results for both species were very similar. It is noticeable that variation about the mean, for individual samples, is generally large. This is most probably due to variations in the efficiency of removing excess water from the soft parts as much as intrinsic fluctuations in condition. The overall trend for both species is a decrease in the condition factor, with time. There is an initial rapid fall in condition for Scrobicularia (Fig.3.5.) over the first 15 days, which is followed by a more gentle decline. This change in the rate of condition factor fall is unexpected because of the constant environment in which the animals were kept. It is unlikely that the values for the first 15 days were a reflection of the acclimatisation to the test tanks, as the animals were given 5 days before the start of any measurements, in order to do just that.

In both cases the curve was fitted by a simple linear regression. The curves indicate a similar decrease in condition factors as the experiments proceed, but Scrobicuaria loses condition at a faster rate ($-0.223\%\ day^{-1}$ Scrobicularia and $-0.168\%\ day^{-1}$ Macoma). These rates result in condition factors for both species falling about 10% - 13% by the end of the 60 day experiment. (N.B. these values are lower than are obtained by comparing only the initial (day 0) condition factors, and those on day 59) Although there were no mortalities, the declining condition suggests that the Macoma and Scrobicularia were under some significant stress. This leads to the possibility of the test animals being more vulnerable to alkyllead exposure because of their degenerating

condition resulting in overestimates of toxicity. As comparisons between the toxicity of specific compounds to acclimated animals, and to ones from the natural habitat are not available, the degree of test-induced vulnerability is unknown. Great care should then be used when expressing relative acute toxicities, as some differences are likely to occur between natural populations and test animals from the present study.

However, the determination of the degree of toxicity pertaining to each alkyllead compound tested is still possible, assuming that all of the test animals maintained similar condition factors. The condition factors for control animals, at the beginning and at the end of each test can be seen in Table 3.1.

For both species there is some variation in initial condition factor between animals from different tests. A high proportion of this variance may be due to handling operations and excess water removal. There is a greater difference between condition factors at the end of the experiments and also therefore, in the percentage differences between start and finish values. It should be noted that each value is a mean of 5 individual measurements of condition factor, and consequently significant variation should be expected. Generally there is a 20%-40% fall in condition factor over the length of the experiments, although a clear relationship between length of exposure and fall in condition factor is not obvious. The exception is in the triethyllead experiment, which was run for 35 days with only a 10% fall in condition of Scrobicularia and a probable slight increase in that of Macoma. The measured fall in condition factor could help to explain the mortality of molluscs from the unexposed (control) tanks, although again this is not a simple relationship.

Condition factors for both species from the two TEL experiments cannot be compared directly to the others because TEL test conditions did not

Table 3·1 CONDITION FACTORS FOR CONTROL MACOMA (M) AND SCROBICULARIA (S) AT THE START AND FINISH OF ALKYLLEAD TOXICITY BIOASSAYS.

(Each value is the mean of 5 individuals ± 1 standard deviation)

Compound Tested	Species	Condition Factor Start	Finish	Change in C.F. %	Length of expt. days	% dead
control test	M	41·8 ± 9·6	30·1 ± 7·4	-28·0	60	0
	S	49·5 ± 5·2	25·8 ± 8·0	-47·9	60	0
TEL (36µl l^{-1})	M	39·2 ± 9·1	31·2 ± 6·7	-20·4	50	20
	S	39·9 ± 5·8	23·4 ± 9·3	-41·4	50	75
TEL (72µl l^{-1})	M	38·4 ± 7·5	27·8 ± 5·2	-27·6	40	0
	S	41·5 ± 6·1	28·8 ± 7·3	-30·6	18	100
Me_3Pb^+	M	39·8 ± 5·8	25·4 ± 8·8	-36·2	60	5
	S	41·2 ± 6·7	28·9 ± 8·3	-29·9	60	10
Et_3Pb	M	43·6 ± 7·2	43·9 ± 10·5	+0·7	35	0
	S	44·7 ± 5·3	40·1 ± 5·9	-10·3	35	0

include aeration or illumination (due to the highly volatile and photolytic nature of TEL). This harsher environment obviously subjected the animals to excessive stress resulting in unacceptable mortalities in respect to confining any toxic action to TEL. A major reason for the decline in condition in the control tanks lies most probably in food shortage, rather than the effect of the physical environment.

3.5. Seawater Alkyllead Concentration

Difficulties in maintaining steady concentrations of TEL in seawater were expected because of the sparingly soluble, volatile and unstable nature of the material. When possible regular monitoring of all the experiments was undertaken, and detailed values of the analyses can be seen in Tables 3.2. to 3.7. The analyses for triethyl and trimethyl lead combine trialkyl and dialkyl concentrations, although the majority would be in the tri form[5]. The TEL experiments include tri and di measurements also, because of the rapid degradation of TEL to those lower analogues. Usually the replicate tanks showed similar concentrations and, where no significant difference was found between the two, both tanks were used for a single mean value. Samples for analysis were taken by automatic pipette, from approximately mid-water depth, care being taken not to collect any surface water which may have supported a thin layer of TEL.

Some of the variation can be attributed to experimental errors in the analytical procedure (see 2.4.1.1.), although analyses of known concentrations usually gave values within 10% of those expected. It is most probable, then, that the values recorded were close to the actual concentrations present. In all of the experiments, except 72 µl TEL l^{-1}, there was regular changing of the seawater. This procedure could have enabled a build-up of concentration of the various lead compounds, due to poor efficiency in interim tank washing, or resolublisation of adsorbed molecules. However, these sources of contamination were checked by analysing fresh seawater placed in freshly cleaned tanks, that had previously contained triethyllead or TEL. Any alkyllead present in this seawater was at concentrations below the detection limit of the analytical method (0.003 mg l^{-1}). Presumably any adsorbed material would require solvent extraction to release it[5].

TABLE 3.2 $[Et_3Pb^+ + Et_2Pb^{++}]$ IN SEAWATER CONTAINING SCROBICULARIA EXPOSED TO VARYING $[Et_3PbCl]$ $(mg\,l^{-1})$

Time (days)	Time of seawater changes	Control	0·15		0·30		1·00	
			a	b	a	b	a	b
0	2	<0·003	0·12	0·16	0·25	0·19	0·91	0·93
3	4		0·15	0·13	0·25	0·31	0·96	1·21
7	7 9		0·13	*0·04	0·15	0·18	0·96	1·16
14	11 14	0·004	0·16	0·15	0·28	0·28	0·92	1·00
21	16 18	<0·003		0·14				
28	21 23 25	<0·003		0·14				
Mean		<0·003	0·14		0·24		0·94	1·08
±1 S.D.			0·01		0·06		0·03	0·13

TABLE 3.3 $[Et_3Pb^+ + Et_2Pb^{++}]$ IN SEAWATER CONTAINING MACOMA EXPOSED TO VARYING $[Et_3PbCl]$ $(mg\,l^{-1})$

Time (days)	Time of seawater changes	Control	0·15		0·30		1·00	
			a	b	a	b	a	b
0	2	<0·003	0·13	0·16	0·30	0·34		1·17
3	4		0·10	0·09	0·24	0·33		1·60
7	7 9	<0·003		0·12	0·96	0·24	*2·56	
14	11 14 16			0·13	0·34		1·60	1·70
22	18 21	0·010	0·10	0·14	0·30		1·81	1·51
29	23 25	<0·003	0·15	0·14	0·28			
Mean		<0·003	0·13		0·30		1·56	
±1 S.D.			0·02		0·04		0·22	

TABLE 3.4 $[Me_3Pb^+ + Me_2Pb^{++}]$ IN SEAWATER CONTAINING SCROBICULARIA EXPOSED TO VARYING $[Me_3PbCl]$ ($mg\ l^{-1}$)

Time (days)	Time of seawater changes	Control	0·05		0·10		1·00	
			a	b	a	b	a	b
0	3							
7	7	0·004	0·08	0·10	0·13	0·12	1·50	1·26
10	10 14	<0·003	0·07	0·07	0·08	0·11	1·01	1·09
20	17 21		0·11	0·09	0·12	0·11	1·16	1·46
31	24 28 31	0·004	0·09	0·07	0·10	0·12		
55	35 42 38	<0·003	0·08		0·11			
Mean	46 50	0·004	0·08		0·11		1·25	
±1 S.D.			0·01		0·02		0·20	

TABLE 3.5 $[Me_3Pb^+ + Me_2Pb^{++}]$ IN SEAWATER CONTAINING MACOMA EXPOSED TO VARYING $[Me_3PbCl]$ ($mg\ l^{-1}$)

Time (days)	Time of seawater changes	Control	0·05	0·10	0·50	3·00	6·00
0	3	< 0·003	0·09	0·12	0·51	3·2	6·0
7	7		0·05	0·10	0·40	3·0	7·2
10	10 14	0·004	0·01	0·09	0·41	3·5	6·5
20	17 24 28	0·004	0·01	0·15	0·44		
31	31 38 35 42	< 0·003	0·04	0·14			
55	46 50	< 0·003	0·03				
Mean		< 0·003	0·04	0·12	0·44	3·2	6·6
±1 S.D.			0·03	0·03	0·05	0·3	0·6

TABLE 3.6 [TEL] AND [Et_3Pb^+/Et_2Pb^{++}] IN S.W. CONTAINING BIVALVES EXPOSED TO 72µl TEL l^{-1} ($mg\ l^{-1}$)

Time (days)	Control	Scrobicularia				Macoma			
		TEL		Et_3Pb^+/Et_2Pb^{++}		TEL		Et_3Pb^+/Et_2Pb^{++}	
		a	b	a	b	a	b	a	b
0		0·115		0·070		0·105		0·725	
1	< 0·003	0·014		0·085		0·280	0·070	0·008	0·018
2		0·010	*3·4	0·195	*0·215	0·165		0·045	
3	< 0·003	0·205		0·053		0·020		0·080	
5		0·015	0·055	0·009	0·046	0·005	0·085	< 0·003	< 0·003
6		< 0·003	0·240	0·105	< 0·003	0·035		< 0·003	
7		< 0·003	0·013	0·110	< 0·003	0·034	< 0·003	0·041	< 0·003
8	< 0·003	0·004	0·018	0·005	0·065	< 0·003	< 0·003	0·004	0·054
9		0·040	< 0·003	0·004	< 0·003	0·005	0·010	0·025	0·015
15			< 0·003		0·030	< 0·003	< 0·003	0·013	0·018
20						< 0·003	0·004	0·009	0·010
27	< 0·003					0·009	0·006	0·220	0·044
Mean	0·003	0·045	0·055	0·071	0·025	0·056	0·023	0·098	0·021
±1 S.D.		0·070	0·092	0·062	0·027	0·087	0·034	0·207	0·019

TABLE 3.7 [TEL] AND [Et_3Pb^+/Et_2Pb^{++}] IN SEAWATER CONTAINING BIVALVES EXPOSED TO 36µl TEL l^{-1}

Time (days)	Water change times	Control	Scrobicularia				Macoma			
			TEL		Et_3Pb^+/Et_2Pb^{++}		TEL		Et_3Pb^+/Et_2Pb^{++}	
			a	b	a	b	a	b	a	b
1			< 0·003	0·018	< 0·003	0·008	< 0·003	0·012	< 0·003	0·004
3	3	< 0·003	0·020	< 0·003	< 0·003	0·150		0·232		0·055
6	6		0·075		0·045		0·220	0·400	0·120	< 0·003
8	8	< 0·003	< 0·003	0·015	0·023	0·012	< 0·300	0·014	0·024	< 0·003
9	10			0·070		< 0·003	*14·5	< 0·003	0·040	0·045
15	12 15	< 0·003	< 0·003	0·004	0·004	0·009	0·013	< 0·003	0·011	0·016
16	17		0·011	*2·100	0·026	0·020	< 0·003	0·132	0·008	0·016
21	20 22			0·130		0·006	0·016	0·010	0·018	0·023
30	24 27 30 31	< 0·003	0·013	< 0·003	0·017	0·011		< 0·003		0·009
39	34 36 39		< 0·003		0·012		0·210	0·075	0·012	0·050
45	43	< 0·003	0·004		0·080		0·015	< 0·003	0·046	0·005
Mean		< 0·003	0·015	0·035	0·024	0·027	0·073	0·081	0·031	0·021
±1 S.D.			0·020	0·048	0·025	0·050	0·108	0·129	0·036	0·020

The mean values of exposure for each tank have been calculated to enable easier comparisons between different alkyllead compounds and animals. This is justifiable, as concentrations did not vary greatly with time. The times of seawater change are given in Tables 3.2. to 3.7. When seawater changes were made, clean tanks were also used to enable washing of the exposed ones. Washing consisted of de-leading the glassware with 50% nitric acid for 24 hours followed by thorough rinsing in tap water, until all traces of the acid had disappeared (this was established by a steady state pH).

The initial TEL experiments (72 $\mu l\ l^{-1}$), did not include any changes in the seawater, to minimise the risks of TEL exposure to the handler. Consequently a rapid decline in the system was observed, with noticeable bacterial growth after 9 days. The experiment provides information about the behaviour of TEL in an enclosed, small volume of seawater even though the biological results are of little use. In an attempt to improve the quality of biological data, a follow-up experiment was undertaken under similar conditions, other than that the volume of TEL added was halved (36 $\mu l\ l^{-1}$), and the seawater was changed every few days. By changing the seawater it was hoped that dissolved oxygen concentrations would remain higher, reducing bacterial growth and imposing less stress on the animals. The concentrations of TEL decomposition products could not, therefore, build-up as in the first experiment, although such a noticeable rise is not obvious.

The means calculated from the second experiment show a large variation of concentrations within each tank. Many of the values are near to the detection limit of the analytical procedure, so their accuracy must be treated with caution. A few values are outstandingly high, well above the solubility of TEL, and these are probably due to contamination by liquid TEL in suspension, or from the seawater surface. These values

are not included in the means. The overall results suggest soluble TEL concentrations of 0.015 mg l^{-1} to 0.081 mg l^{-1}, which are below the solubilities given for TEL in the literature[2,3,5,102]. The results for combined trialkyl and dialkyl lead concentrations are of a similar order, 0.021 mg l^{-1} to 0.031 mg l^{-1}, suggesting little decomposition of TEL.

3.6. Toxicity of Trialkyllead Compounds to Macoma and Scrobicularia

All of the toxicity tests with Macoma and Scrobicularia followed the same general pattern, as can be seen in Figs 3.7,3.8,3.9 and 3.10. For the first 5-10 days of exposure there is a relatively low rate of mortality, after which there is a rapid increase in the numbers of deaths. This rate then tends to fall off, giving the cummulative mortality curves an almost sigmoidal appearance. Trialkyllead control tank mortalities were, on the whole, well below 10% so that, although the condition of the animals was declining, mortality in exposed tanks was most probably a result of alkyllead toxicity.

The initial low rate of mortality is possibly a result of the previously discussed avoidance behaviour observed in both species. Observations of the animals revealed that more animals in exposed tanks exhibited valve closure than in control tanks. However, this behaviour was noticeably absent in several exposed animals. It appeared that the animals were better at reacting to changing conditions, rather than maintaining a response for long periods under constant conditions. The periods of valve adduction were not quantified, but are estimated to be in the region of several hours rather than days. Animals exposed to alkyllead were, therefore most probably in contact with the material, which may have resulted in avoidance behaviour for short periods. Whether this alone can explain the slow initial mortality rate is doubtful. If temporary valve closure was used as avoidance initially, it seems that such

FIG 3.7
MORTALITY OF SCROBICULARIA EXPOSED TO VARYING [Me_3Pb^+]

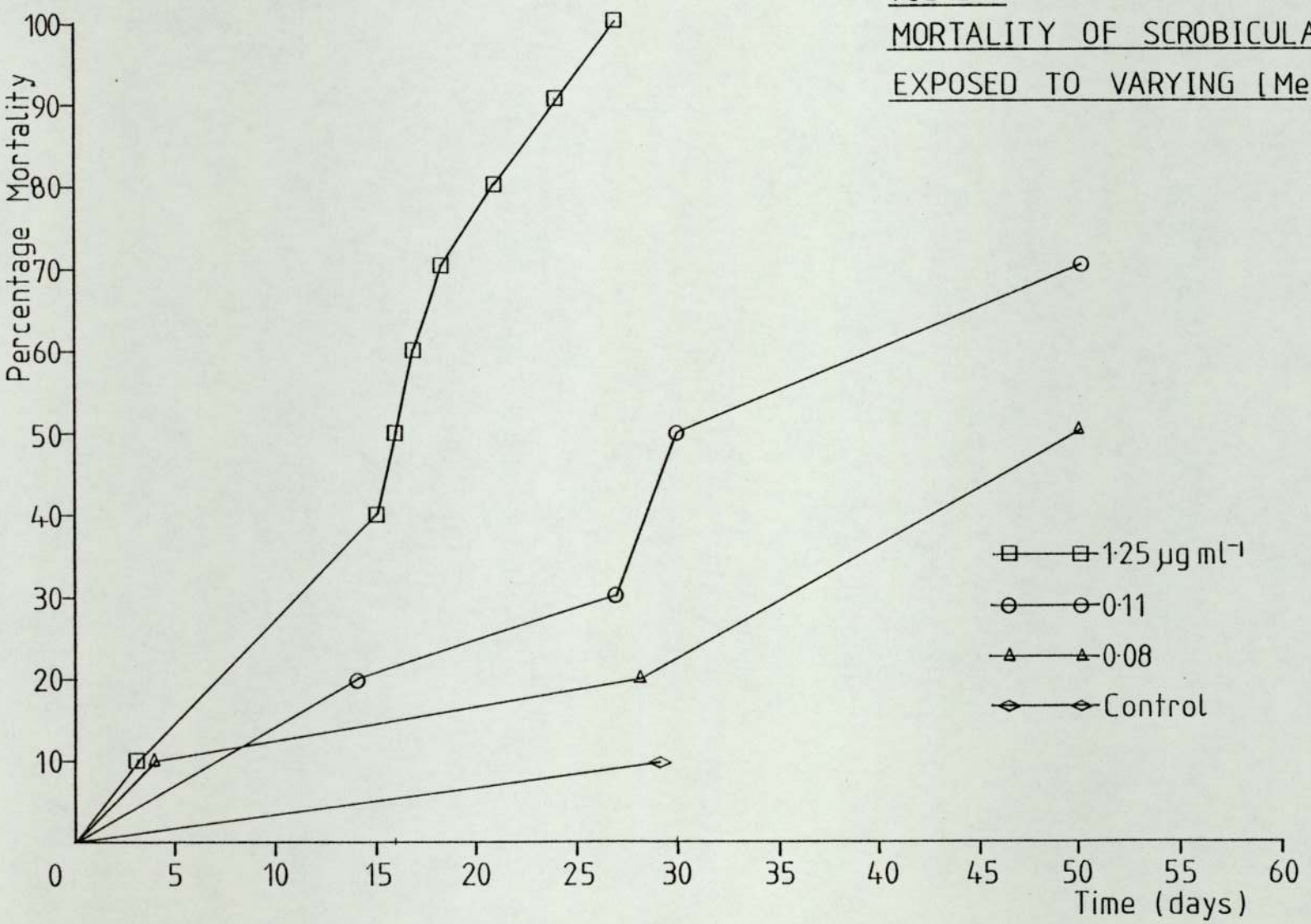
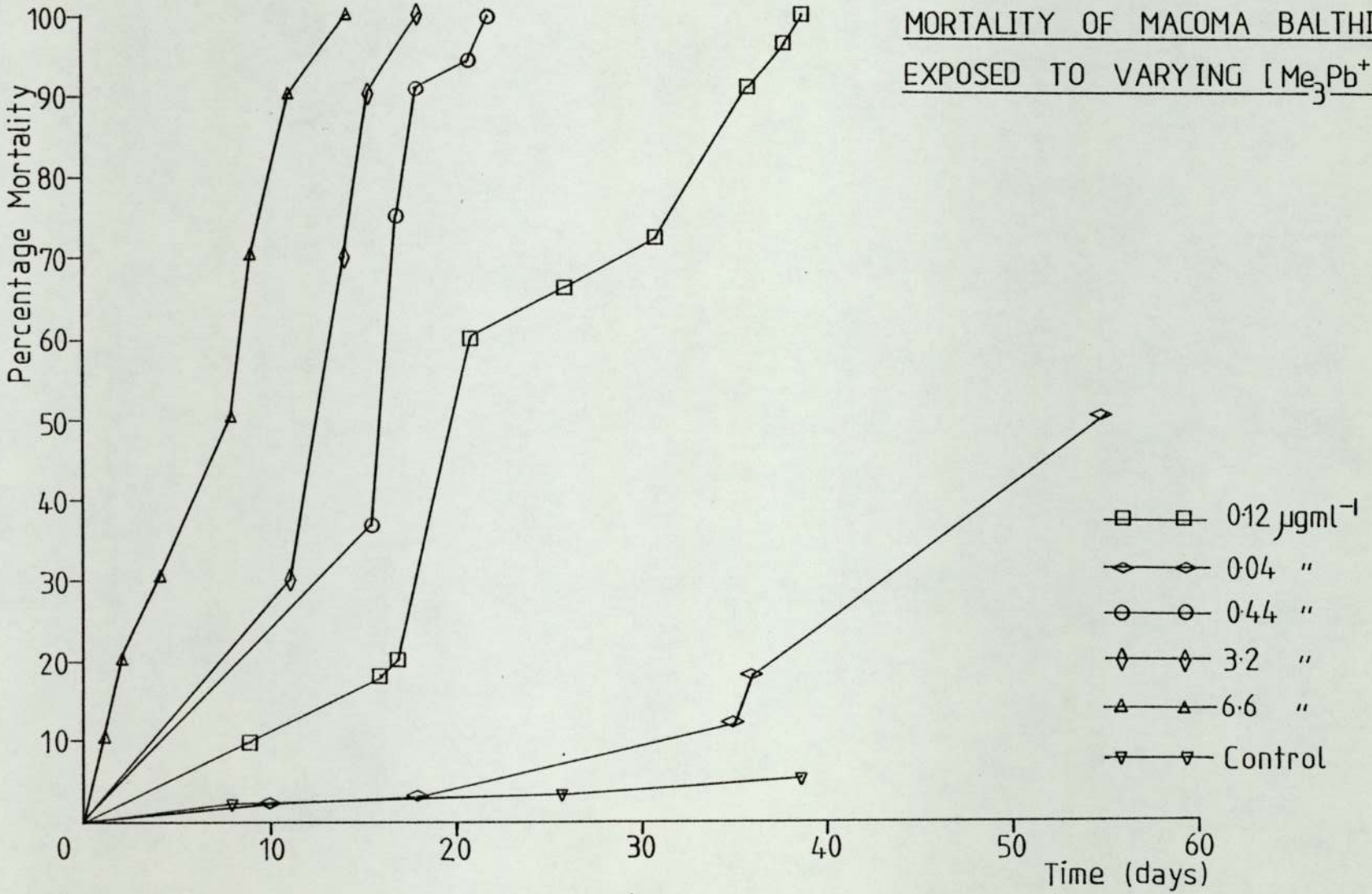


FIG 3.8

MORTALITY OF MACOMA BALTHICA EXPOSED TO VARYING [Me_3Pb^+]



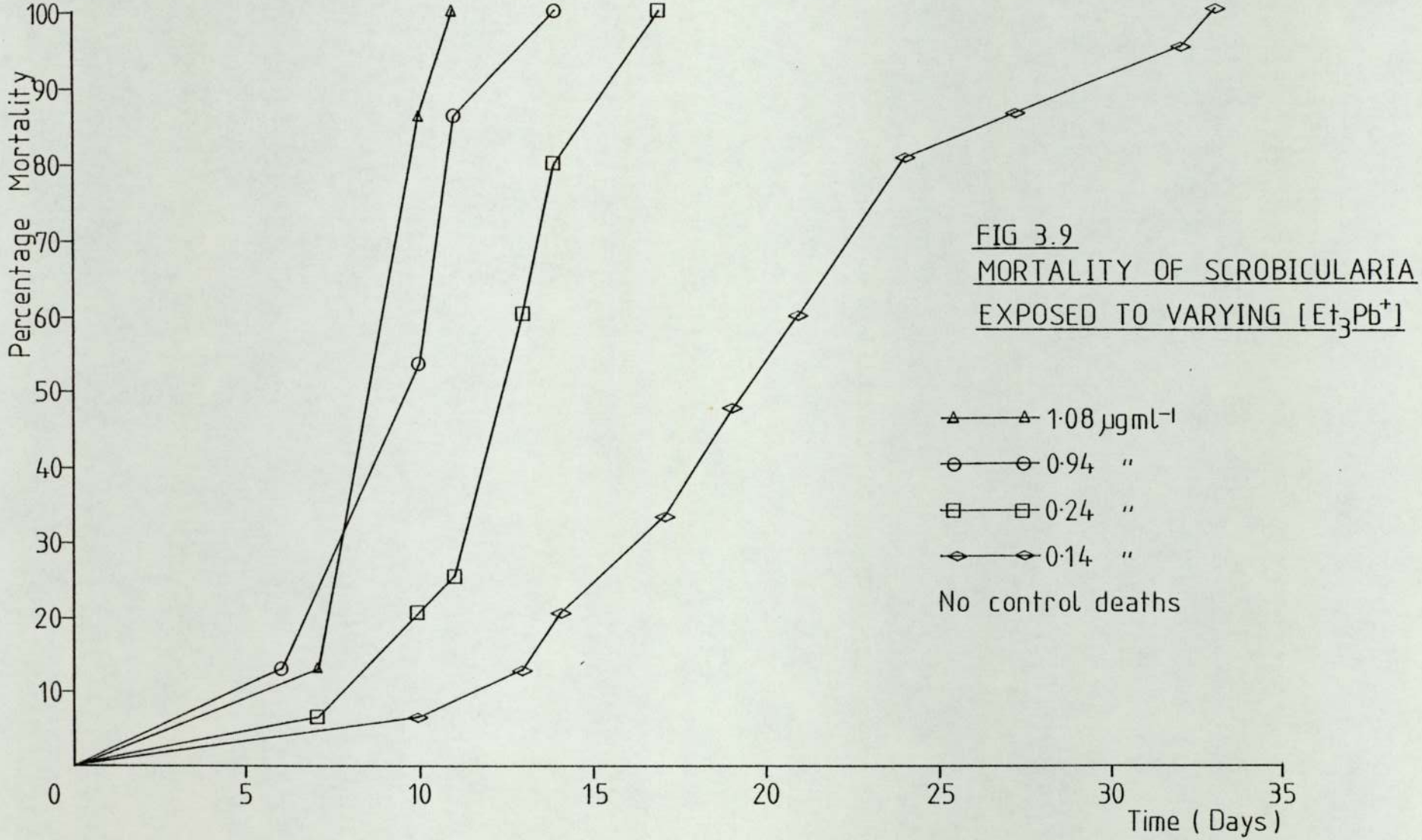
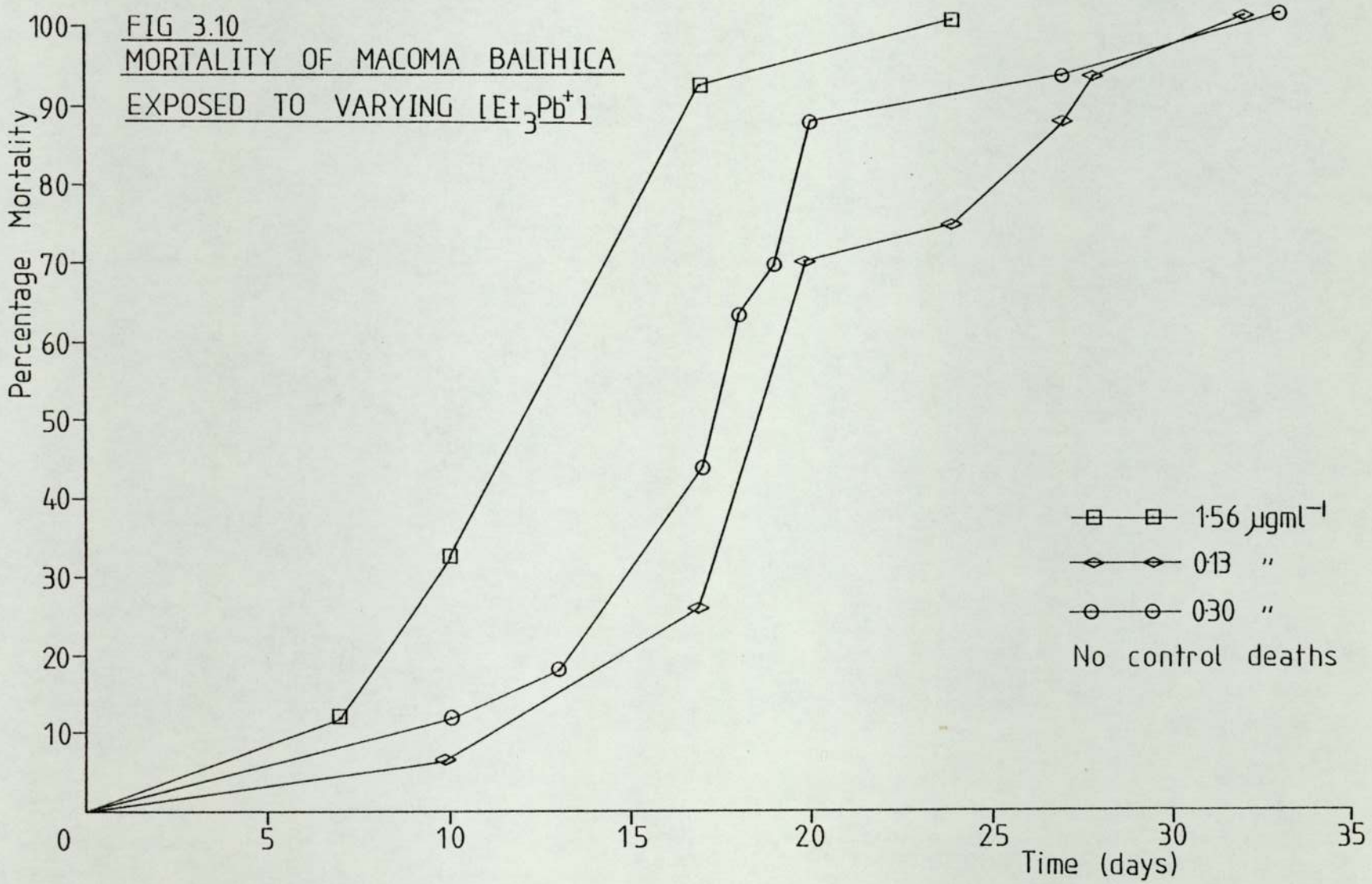


FIG 3.9
MORTALITY OF SCROBICULARIA EXPOSED TO VARYING $[Et_3Pb^+]$

FIG 3.10
MORTALITY OF MACOMA BALTHICA
EXPOSED TO VARYING [Et_3Pb^+]



behaviour could continue indefinitely, as long as intermittent feeding and aerobic respiration also continued. An accurate explanation of the lag in mortality is probably highly complex, involving aspects of the behaviour and also the physiological condition of exposed animals.

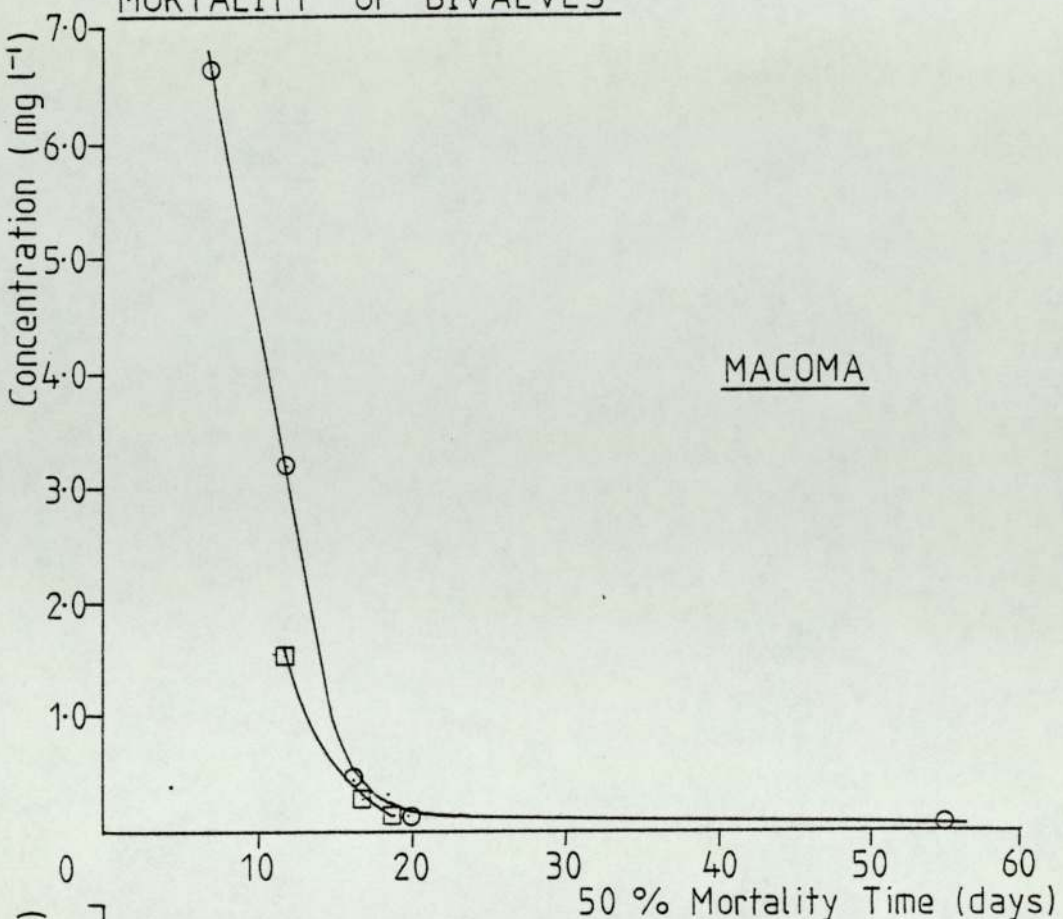
Because of some difficulties in maintaining specific concentrations of both trialkylleads, comparisons of toxicity at one concentration were not possible. However, by plotting the relationship between concentration and 50% mortality periods (Fig.3.11), with the limited data available, comparative toxicity can be discussed more easily. Triethyllead chloride appears more toxic than trimethyllead chloride over the whole range of possible comparisons in Fig.3.11, although the Macoma data are less clear. With the limited data, an exact measure of the greater toxicity of the ethyl compound over the methyl compound is difficult. An approximate estimate lies in the range 1.5 - 4.0 for Scrobicularia and 1.9 - 2.1 for Macoma.

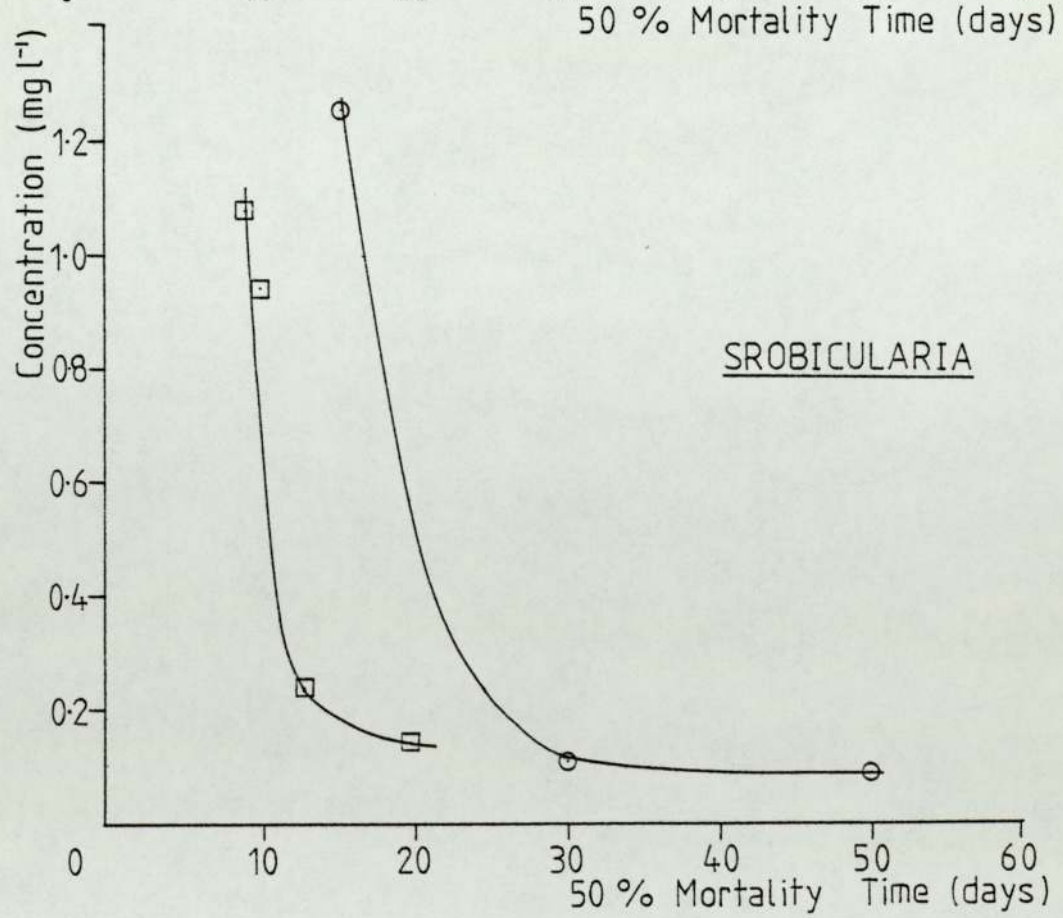
The relationship is not linear over the whole concentration range studied. Each curve shows a sudden, rapid change in gradient at particular concentrations. For Scrobicularia exposed to concentrations below 0.2 mg l^{-1} of both trialkyls, small concentration changes result in large changes in the time taken for 50% of the population to die. Above these concentrations the reverse is true, i.e. large concentration increments result in only small time reductions. Similar changes in gradient for Macoma occur at approxiamtely 0.3 mg l^{-1} for trimethyllead chloride and lower for triethyllead chloride.

The changing slope of all curves suggests that there may be a threshold concentration for each trialkyllead compound, above which toxicity increases only slightly , but below which decreases rapidly. By extrapolating the curves towards the x-axis, a point should be reached

FIG 3.11

RELATIONSHIP BETWEEN CONCENTRATION OF Et_3Pb^+ (⊟—⊟) AND Me_3Pb^+ (⊖—⊖) AND TIME TO 50% MORTALITY OF BIVALVES





where a minimum concentration just produces a 50% mortality rate after an indefinite exposure period. This is the incipient exposure concentration[51] which can be reduced by an appropriate factor (0.1 - 0.001) to give safe limits. There are insufficient data to enable incipient concentrations to be calculated accurately but tentative values for trimethyllead chloride are 0.04 mg l^{-1} for Macoma and 0.08 mg l^{-1} for Scrobicularia. By applying 0.1 as an appropriate factor[51], approximate safe levels of 0.004 mg l^{-1} for Macoma and 0.008 mg l^{-1} for Scrobicularia are reached. The data for triethyllead chloride are insufficient for similar calculations.

From these calculations, and from interspecific comparisons of concentrations required for 50% mortality, it appears that Macoma are less tolerant than Scrobicularia to trimethyllead chloride but more tolerant to triethyllead chloride. This would be easier to explain if either species were consistently more tolerant than the other, to both compounds. Both compounds have similar chemistry, so why Macoma finds trimethyllead more toxic than Scrobicularia but triethyllead less, is extremely puzzling. A larger set of data would help to consolidate these findings, but is not presently available.

No concentration produced 50% mortality within a 96 hour period, so that comparisons with LC_{50} values cannot be made. However, the relative toxicities can be compared. Maddock and Taylor[51] found, in general, ethyl derivatives to be more toxic than methyl derivatives. But in the only bivalve they tested (Mytilus), trimethyllead chloride was twice as toxic as triethyllead chloride. This underlines the difficulties in comparing different responses in different species. Meaningful comparisons of relative toxicities can only be made between individuals of one species, with identical physiology and behaviour. Most of the literature on alkyllead toxicity to aquatic organisms also supports the

general higher toxicity of ethyl groups over methyl groups, but no further data on bivalve toxicity other than that of Maddock and Taylor[51] has been found.

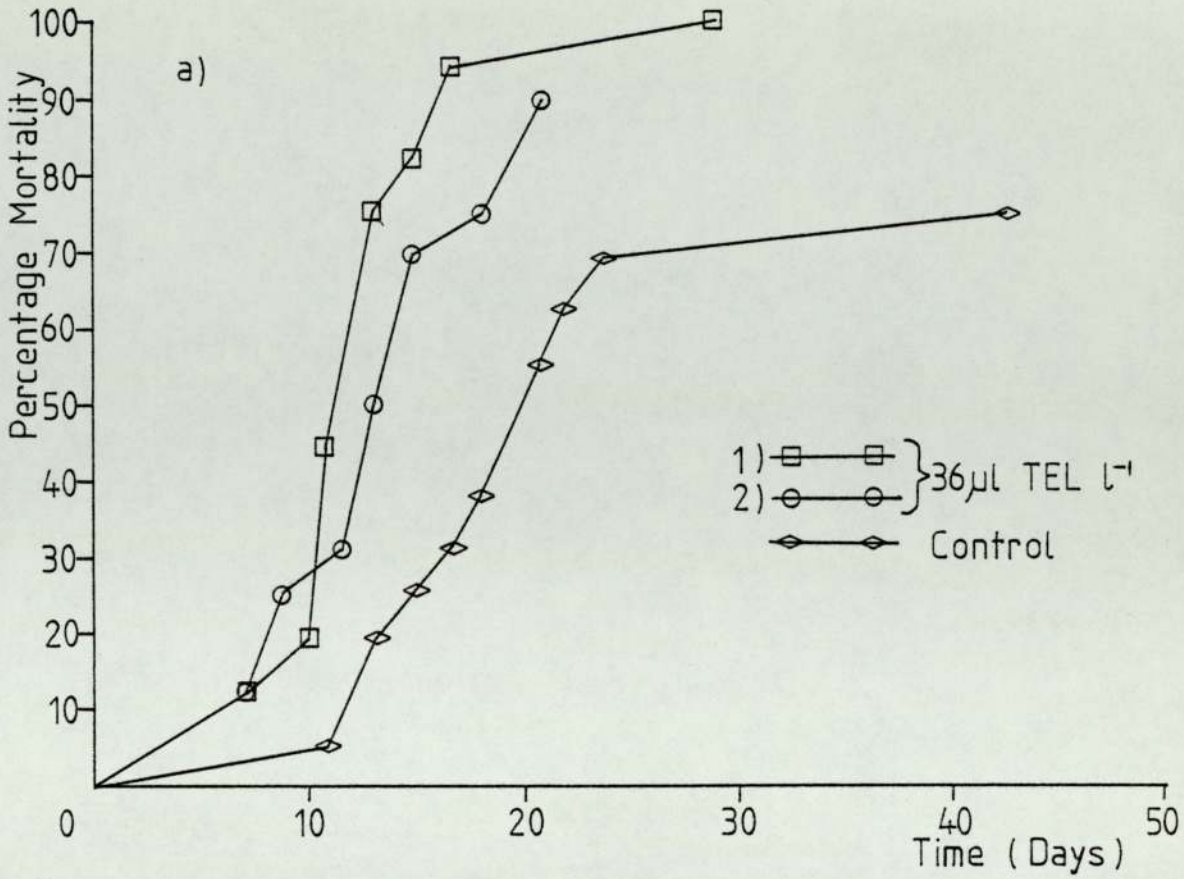
3.7. Toxicity of TEL to Macoma and Scrobicularia

The difficulty in testing TEL toxicity was to ensure animal exposure, but at the same time preventing the material from leaving the test system, thereby endangering anyone in the vicinity (i.e.in the laboratory). It was not possible to maintain the test tanks in a fume-cupboard, so TEL losses by spillage of exposed seawater or volatilization had to be prevented. The test vessels were therefore, required to be closed, as much as was possible, prohibiting aeration and minimising seawater changes. These two limitations resulted in 72 μl TEL l^{-1} initial experimental environment, rapidly degenerating towards anoxia and tremendous bacterial growth. Considerable bacterial growth was observed in all Scrobicularia tanks, after 9 days, including the controls (Fig.3.12b). All of the TEL exposed Scrobicularia were dead after 14 days and 100% mortality was reached on day 17. The major factor causing mortality must be assumed to be the highly inhospitable environment, any TEL action being hidden by the rapid control mortality.

In contrast to these larger animals, no mortalities of the smaller Macoma were registered under the same conditions. It was noticeable that bacterial contamination was of a far smaller degree, suggesting higher oxygen concentrations than in the Scrobicularia tanks. This was probably due to the smaller size of the Macoma, using oxygen at a slower rate, even though there were a quarter more individuals than in the Scrobicularia tanks (20 vs 15). There was also a marked difference in the mortality rate of the TEL exposed animals (Fig.3.13b). Both tanks produced a low rate initially, as in the majority of tests, but after day 10 replicate 2 registered a rapid increase, reaching 90% mortality after 18 days.

FIG 3.12

MORTALITY OF SCROBICULARIA PLANA EXPOSED TO TEL



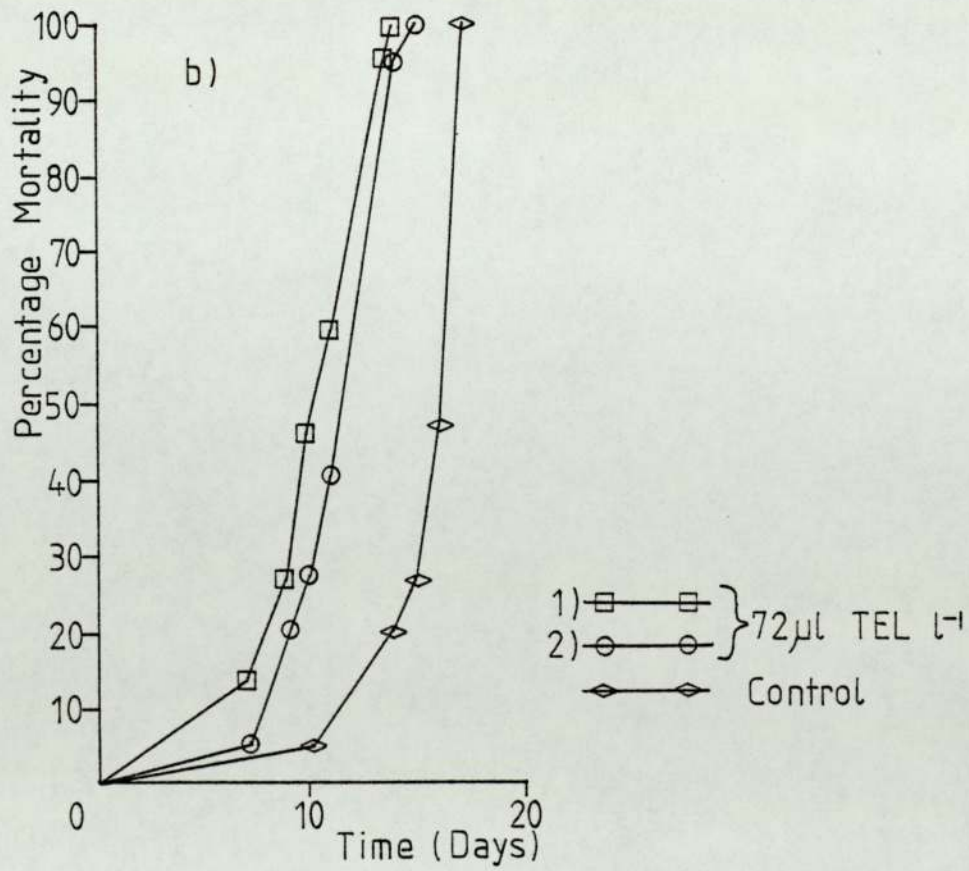
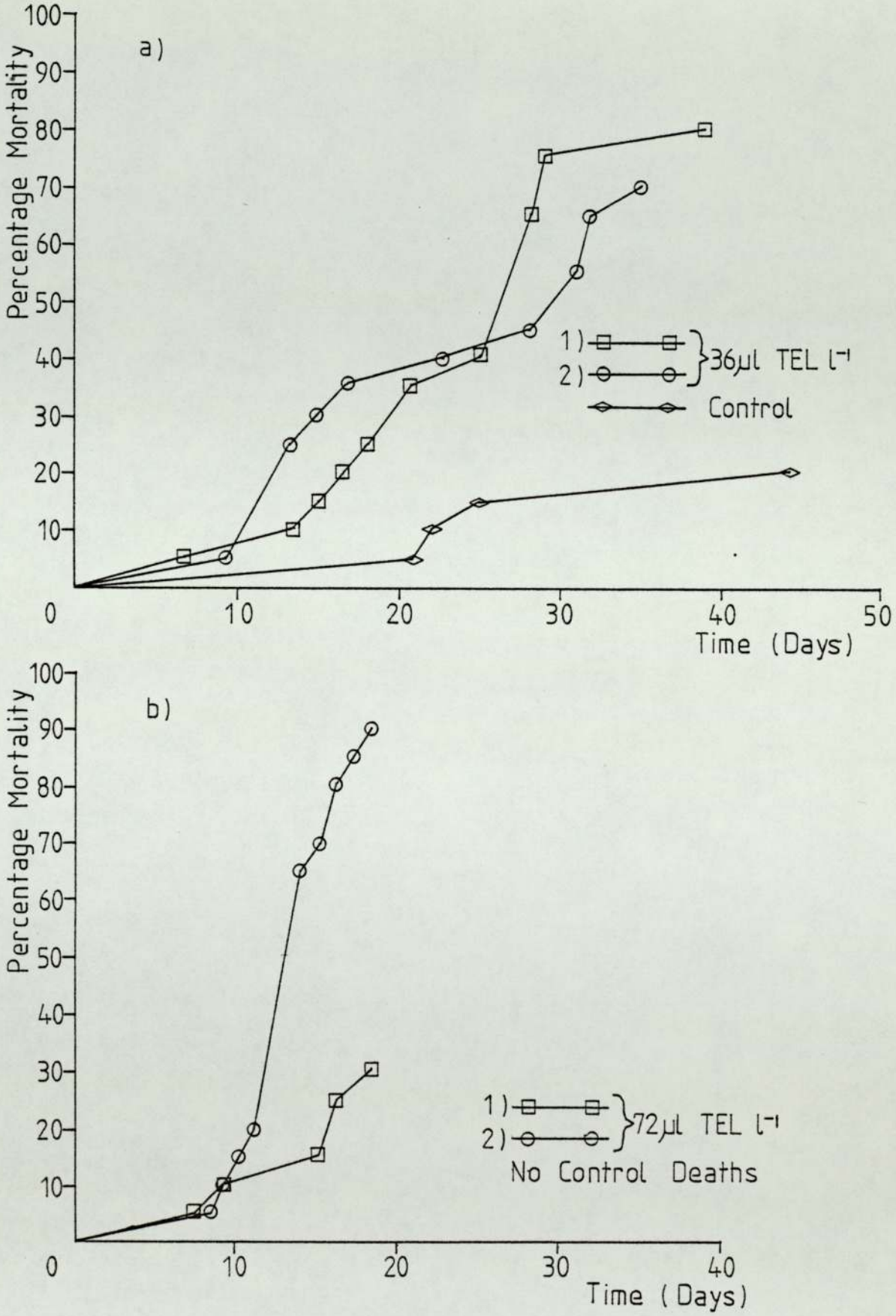


FIG 3.13

MORTALITY OF MACOMA BALTHICA EXPOSED TO TEL



No further mortalities were observed over the remaining duration of the experiment (22 more days). The absence of any deaths after 18 days may be related to the very low concentrations of TEL detected in the latter part of the experiment. The combined trialkyl and dialkyl lead degradation products, on the other hand, show a general increase in the concentration throughout the exposure period, although the maximum values reached are not likely to be highly toxic alone (compare with Fig.3.10.). The low TEL concentrations may have been the result of glass adsorption and loss of TEL by volatilisation, although liquid TEL was observed at the bottom of all TEL exposed tanks.

The data gained from Scrobicularia tanks cannot be used to estimate the toxicity of TEL but the Macoma data are more useful, as no control deaths occurred. Even so, any measure of toxicity is likely to be highly inaccurate because of the poor conditions within the tanks. These conditions are reflected in the great deterioration in condition factor (Table 3.1.) of control Macoma, over a relatively short period. However, the most likely cause of the mortalities in both Macoma replicates, is the action of TEL or its decompostion products, or both. The absence of any deaths after day 18 suggests TEL as the main agent, as this is commensurate with the falling concentration detected. But these low TEL values were recorded from 7 days onwards, well before the lethal action ceased. Perhaps the mainly trialkyllead decompostion product was the cause, for even at the low concentrations determined the deleterious conditions may have acted synergistically with it, to kill the animals. This does not explain the sudden cessation in mortality.

Both explanations, therefore, have contradictions and with the poor quality of biological data, it is difficult to draw any useful conclusions. Frequent changes in seawater, every 2 - 3 days, in the second TEL bioassay, helped to prevent noticeable bacterial infestation

of any tank, and presumably maintain higher oxygen saturations. It also avoided a build-up of triethyllead from TEL decomposition, so that any alkyllead toxic action should have been entirely due to TEL. Condition factors for both species again show a marked decline, although Macoma appeared to maintain condition to a greater extent than in the first bioassay (Table 3.1.).

Again the mortality rate of Scrobicularia control animals is high, reaching over 70% in 24 days. This is possibly due to the same causes that applied to the earlier tests. The replicate exposed populations also showed rapid mortality rates, with one (1) reaching 100% after 29 days. The other replicate (2) did not exceed 90%, although it showed an almost linear increase in this value over 21 days. It cannot be concluded that the lower number of deaths was due to lower TEL concentrations present (Table 3.7.), or the absence of trialkyllead build-up because of the unacceptable control mortality. Indeed the reason probably lies in the less anoxic conditions imposing less stress on control and exposed tanks.

The Macoma data are also poor, with 20% mortality after 44 days. Why no controls died in the earlier tests but did in this less harsh environment is puzzling, especially considering the condition factors obtained. Both exposed replicates showed similar mortality rates, reaching 70% to 80% after 35 to 39 days. No further deaths were recorded. The control mortality rates in this bioassay, being well below the exposed rates, suggest that there may be some toxic action related to alkyllead. Concentrations of Et_3Pb^+ and Et_2Pb^{++} were similar to the 72 $\mu l\ l^{-1}$ values, again making it difficult to confer TEL toxicity

On the whole, this bioassay method for determining TEL toxicity to Macoma and Scrobicularia is unsatisfactory, partly because of safety constraints and partly because of unsuitable facilities. The only other study to

determine TEL toxicity to bivalves[51] suggests that Mytilus edulis exposed in a flow-through system were more susceptible to TAL than the trialkyllead counterparts. Unfortunately Mytilus mortality rates cannot be directly compared with this study for TAL toxicity, but more general comparsions can be made with the data obtained from the siphonal preparation experiments described in Chapter 4.

3.8. Accumulation of Trialkyllead in Scrobicularia and Macoma

During the course of the previously described exposure of both species to triethyl and trimethyl lead chloride, analyses of the animal lead content were performed. As soon as individual animals died, they were removed from the exposure tank, drained of excess seawater and then labelled and stored in small polythene bags at -18°C. If no animals had died by the end of the exposure period, usually in control tanks only, a suitably sized random sample of live animals was taken. Storage time was never greater than two weeks.

The analytical procedure is outlined in 2.4.2. All samples were determined by AAS and therefore ICl/dithizone extraction was required, after the initial extraction by toluene and nitric acid. Because of this multistage method, detection limits were reduced in comparison with those for water analysis. The preparation of standards, to calibrate the procedure consisted of extracting known aqueous solutions of trialkyllead through the entire sequence, but the variability in these results raised the detection limit to 0.02 mg l^{-1}. The efficiency of alkyllead extraction from water samples (i.e. standards), is obviously greater than that from biological samples. Therefore, most values quoted are probably underestimates of the concentrations actually in the tissues. Extraction efficiency for the whole procedure using biological samples is probably just less than 70% - 80%[57].

All extractions were conducted on a wet weight basis. For the whole body determinations, where more than one animal was available for analysis, all individuals were homogenised and a suitable sub-sample was taken. This allowed several mean determinations of the whole sample rather than a similar number of determinations from fewer individuals.

Distribution of alkyllead compounds in particular organs was only determined for Scrobicularia. Macoma proved too small to supply sufficient single tissue types, even when many animals were massed together. Single Scrobicularia did not yield enough material, ideally about 1.0 gram, so all values are means of several individuals.

The accumulation of trimethyllead by Scrobicularia was not investigated as this bioassay was conducted at a time when biological analysis was not possible due to a shortage of instrument facilities. This also applies to the 6.6 mg l^{-1} and 3.2 mg l^{-1} trimethyllead exposures of Macoma, which were run at the same time.

Where both replicate tanks of particular concentrations were similar, the animals from both were analysed together. However, in the trimethyl lead chloride exposure of Macoma, insufficient numbers were available to occupy replicate tanks.

It should be noted that the major source of biological material was from dead animals, a continuous measure of accumulation was not possible. The times when analyses were performed are, therefore, sporadic with unfortunate gaps when no animals died. In some instances, when insufficent numbers had died to enable analysis, other animals which had died shortly before or after were combined to produce enough material. Consequently some of the results are mean values of accumulation over one or two days.

3.8.1. Accumulation of Me_3Pb^+ by Macoma

Values of trimethyllead concentrations in Macoma (Table 3.8.), suggest accumulation does not increase with time, but does with exposure concentration. The data are too limited for a detailed interpretation of how accumulation is related to these factors, but it is surprising that lower values are obtained after longer exposure periods. This trend cannot be explained by falling water concentrations after about 10 days, as analyses show approximately constant values (Table 3.5.). The degree of accumulation from specific concentrations can be compared by calculating tissue concentration factors after particular exposure periods.

$$\text{Thus: Concentration Factor} = \frac{\text{concentration in tissue (mg Kg}^{-1}\text{ wet wt)}}{\text{concentration in water (mg l}^{-1}\text{)}}$$

where concentration in tissue is related to rate of alkyllead uptake minus rate of alkyllead loss. For all tissue concentration increases, uptake must exceed loss, and at steady state concentrations uptake and loss rates are equal. Accurate determinations of concentration factors, after an arbitrarily chosen time, are difficult because of the widely scattered data. However, approximations of such factors after 20 days suggest a value of 10 - 40x for all exposure concentrations.

3.8.2. Accumulation of Triethyllead by Scrobicularia and Macoma

For both species accumulation appears to increase with exposure concentration and with time (Tables 3.9. and 3.10.). This latter relationship is in contrast with the accumulation of trimethyllead by Macoma. However, it seems more reasonable to expect an increase, or steady state concentration (if a threshold concentration is achieved), if seawater values are constant. Concentrations are again approximately in the range 15 - 50x for both species after 20 days exposure.

Care must be exercised when discussing these results because of the

TABLE 3.8 ACCUMULATION OF Me_3Pb^+ BY MACOMA EXPOSED TO VARIOUS [Me_3PbCl] (µgPb/g.wet wt.)

( )-number of individuals analysed

Time (days)	Mean exposure concentration ($mg\ l^{-1}$) ± 1 S.D.			
	Control	0·04	0·12	0·44
16				9·2 ± 1·2 (11)
18			5·2 ± 0·5 (8)	8·2 ± 0·7 (11)
21				6·4 ± 0·5 (8)
23			1·2 ± 0·3 (10)	
35		0·54 ± 0·18 (4)		
38	< 0·02 (10)		1·5 ± 0·3 (12)	
56	< 0·02 (10)	0·50 ± 0·09 (13)		

excessive stresses suffered by the animals, resulting from exposure to lethal alkyllead concentrations. Anomalies in the rate of accumulation might be expected as the animals are not in their normal physiological or behavioural state. The decline in condition index of control animals suggests that metabolically influenced processes, such as accumulation, may be 'abnormal', and the observed behavioural responses to alkyllead exposure i.e. temporary valve closure and reduced pumping activity, might also lead to the same. However the results may be useful in predicting the likely concentrations of these compounds that can be

TABLES 3.9, 3.10 ACCUMULATION OF Et_3Pb^+ BY MACOMA AND SCROBICULARIA EXPOSED TO VARIOUS [Et_3PbCl]

(μgPb/g.wetwt.) ± 1 S.D.

TABLE 3.9 SCROBICULARIA

Time (days)	Mean exposure concentration ($mg\,l^{-1}$)			
	Control	0·14	0·24	1·08
10		2·1 ± 0·2 (2)		24 ± 4·0 (14)
11			10 ± 1·0 (4)	42 ± 1·7 (3)
13			7·5 ± 0·5 (5)	
14		2·1 ± 0·3 (3)	8·4 ± 1·2 (3)	
23		4·3 ± 0·2 (3)		
34	< 0·02 (6)			

TABLE 3.10 MACOMA

Time (days)	Mean exposure concentration ($mg\,l^{-1}$)			
	Control	0·13	0·30	1·56
10				30 (10)
16		4·4 ± 0·2 (10)	12 (8)	46 (10)
19		6·1 (10)	18 (7)	
24				51 (2)
28	< 0·02 (10)	6·8 (7)	16 (2)	

achieved from high level alkyllead pollution, possibly resulting from a major spill at sea.

3.8.3. Accumulation of Triethyllead in Individual Organs of Scrobicularia

Those organs chosen for analysis were selected by reviewing the relevant literature and also because of the importance of various organ functions. The ciliated gills or demibranchs of lamellibranchs fulfill two major functions, respiration and filtration of incoming particulates for food. The gills therefore receive the maximum aqueous alkyllead loading, as well as handling particles that may include adsorbed alkyllead. The siphons are similarly exposed to aqueous alkyllead, although their surface area is far smaller. The digestive gland receives the greatest loading from particulate associated alkyllead, i.e. lead accumulated by the food source. The rest of the soft tissue was conveniently analysed en masse.

The distribution of triethyllead appears to be spread evenly throughout the animal (Table 3.11.), with the exception of the digestive gland, which accumulated a significantly greater amount of the compound. As the main source of loading was via solution rather than food, this result is surprising. The animals were fed with a diatom, P.tricornutum, but this was cultured in an alkyllead free medium, and could only have accumulated alkyllead from the test tank. As the period between introduction of the algae into the tanks and ingestion by the animals is unknown, though probably short, the degree of algal accumulation may have been significant. Aqueous alkyllead will have contributed to the digestive gland accumulation, but this route would be expected to result in higher gill concentrations.

A more detailed investigation of the alkyllead in food-chain transfer from algae to bivalves is not available, although accumulation in mussels

TABLE 3.11 ACCUMULATION OF Et_3Pb^+ BY VARIOUS ORGAN TYPES OF SCROBICULARIA AFTER 10 DAYS EXPOSURE TO 0·94 mg l^{-1} Et_3PbCl

(µg Pb/g. wet wt.)

	TISSUE TYPE		
Gill	Siphon	Digestive gland	Rest
15	16	42	17
26	23	32	26
34	36	35	22
	26	36	34
		52	28
Mean 25	25	39	25
±1 S.D. 10	8	8	6

exposed to alkyllead in solutions has been studied[51]. In this investigation, Mytilus edulis, after exposure to sub-lethal concentrations of trimethyl and triethyl lead chlorides in seawater were found to have the following relative lead loadings:

Gill > Digestive gland > Foot > Gonad

(In the present study, foot and gonad were included with the remaining

soft tissue that was not individually analysed.)

The mussel gills, as expected, contained the greatest alkyllead concentrations with the digestive gland accumulating more than the remaining tissue. These results are similar to the findings in the present study, with the exception of the gill and digestive gland burdens. The mussels were maintained in a flow-through system with natural seawater, and would have filtered food particles contained in it. The phytoplankton would only be exposed to alkyllead during its passage through the test containers. Dietary alkyllead would presumably be less significant under these conditions than in the present study, which may explain the opposite results.

CHAPTER FOUR

EFFECT OF ALKYLLEAD ON SCROBICULARIA SIPHONAL PREPARATIONS

EFFECT OF ALKYLLEAD ON SCROBICULARIA SIPHONAL PREPARATIONS

4.1. Introduction

There is an enormous range of testing procedures available to determine lethal and sub-lethal effects of chemicals on aquatic organisms[103-105]. Acute toxicity testing, where often the 50% mortality of organisms is taken as an indication of toxicity, is probably the most widely used method of assessing pollutants, (as used in the previous chapter). Chronic concentrations may also ultimately result in mortality, after substantially longer exposures, and useful information regarding growth, reproduction and other sub-lethal responses may be gained during the course of these tests. However, care must be taken when interpreting the results from such experiments, especially long-term assessments. Many factors will be acting upon the test system, apart from those due to the presence of the pollutant. In the present study, because of the facilities available, such tests were unsatisfactory in determining the toxicity of TEL. An alternative procedure was required, which would not involve long periods of exposure. This would overcome the problems encountered with the TEL assays previously described.

Some animals, including bivalve molluscs, can isolate themselves completely from the ambient environment for several days[95]. Thus, actual toxicological mortality may be offset by this behaviour, possibly resulting in misleading interpretations of dose time responses. The widely used LD_{50} or LC_{50} values calculated over 48 hour or 96 hour periods would obviously be greatly influenced by avoidance behaviour.

Valve closure in bivalves is triggered by the sensory apparatus of the animal detecting deleterious conditions. When such conditions prevail, and the valves are closed, periodic environmental sensing allows the mollusc to re-open its valves, and resume pumping activities as soon

as favourable conditions return. It is therefore important to establish the detection limit of the animal, as the concentrations below those detectable may result in sub-lethal toxic effects, bioaccumulation or even mortality. Concentrations above any detection limit would probably cause the valve closure associated with the animals' avoidance behaviour. Methods for determining concentrations of pollutants causing avoidance behaviour in bivalves have been developed by several researchers[95,106-108]. These methods have been principally used with Scrobicularia, although similarly sized deposit feeding lamellibranchs would also be suitable. The first sign of response by Scrobicularia to adverse stress is the rapid retraction of its inhalent and exhalent siphons[108]. Valve closure may then follow, bringing about the complete isolation of the animal. The animal makes contact with the overlying seawater through its siphons, and it is likely therefore, that any sensory apparatus of the siphons will first detect adverse conditions. For this reason it was decided to use a previously tested, siphonal preparation technique[95], to assess alkyllead concentration effects on Scrobicularia. Macoma siphons proved too small and delicate for use in this method, so all experiments were limited to Scrobicularia.

4.2. Siphonal Preparations

The technique can accomodate isolated siphonal preparations, or insitu siphons where the whole of the soft parts are intact. This allows comparison between effects on the siphons alone, and on the whole animal, through siphonal contraction, to be made.

The method applied followed closely to that of Akberali[108]. The animal's shell was opened by slicing through the two adductor muscles, close to the left-side valve. The siphons were then, either excised from the rest of the animal (isolated), or left in place (insitu). In the latter case, the left-side valve was removed by gently easing it

from the mantle tissue with a scalpel. The inhalent siphon tip was ligatured and connected with a fine thread to an isotronic transducer via a pulley system. In isolated preparations the base of the siphon was also ligatured and fixed to a central position in an organ bath by a pin. Insitu siphon preparations were fixed to a similar position by attaching the lower valve with molten black wax. Adjustments to the transducer arm were made to gradually elongate the inhalent siphon to the predetermined length of 3 cm. This length constituted the relaxed and extended state of the siphon and was used as the baseline for all recordings.

All preparations were kept under seawater to achieve minimal contact with the atmosphere. The temperature of the seawater was 10°C, and saturation was achieved by a fine, glass capillary tube, which effectively mixed the seawater and alkyllead solution additions. Complete mixing by this method took about 30 seconds[109].

After the short time to obtain such a baseline, the siphon was mechanically stimulated by squeezing with forceps to elicit a contraction response. On contraction, the transducer arm was pulled down by the thread attached to the siphon. The angle of deflection was directly proportional to the deflection of an oscillograph pen, used for recording. The siphon rapidly returned to its relaxed state and shortly afterwards the various alkyllead additions were made.

Isolated siphonal preparations remained viable for over an hour. In most cases however, recordings were terminated before 60 minutes. This also reduced the problem of the seawater temperature rising above 10°C. The seawater was refrigerated at 10°C, but all experiments were carried out at room temperature. A linear increase in temperature was recorded with time (up to 3°C) after the initiation of the experiment. However, during

washing procedures, fresh seawater was introduced again at 10°C.

4.2.1. Siphonal Responses to Alkyllead Compounds

The experiments were conducted during February and July of 1981 in the Zoology Department of Manchester University, and in October of 1981 at Aston University. Identical experiments carried out during each of these periods produced similar results. No seasonal or interlab. variations were observed. Alkyllead additions to the organ-bath containing siphonal preparations in artificial seawater were made by automatic pipette, in order to obtain precalculated concentrations. The tri and di- alkyl lead salts were dissolved in artificial seawater or distilled water, if the former medium prevented effective dissolution. When distilled water was used, stock concentrations were maximised to enable small volumes of stock to be added to the preparations, thereby minimising salinity changes. The maximum salinity change incurred from these dilutions was 6%. In such cases, additions of similar volumes of distilled water only, were added to the organ-baths, but no siphonal contractions occurred. Thus, any response that occurs can be assumed to be due to the action of alkyllead present in the medium, as all other conditions in these experiments except salinity were constant. The alkyllead stock solutions used were analysed with known standards by the AAS method as outlined in Chapter 2. All concentrations in the organ-bath were calculated from the dilution factors of the known stock concentrations.

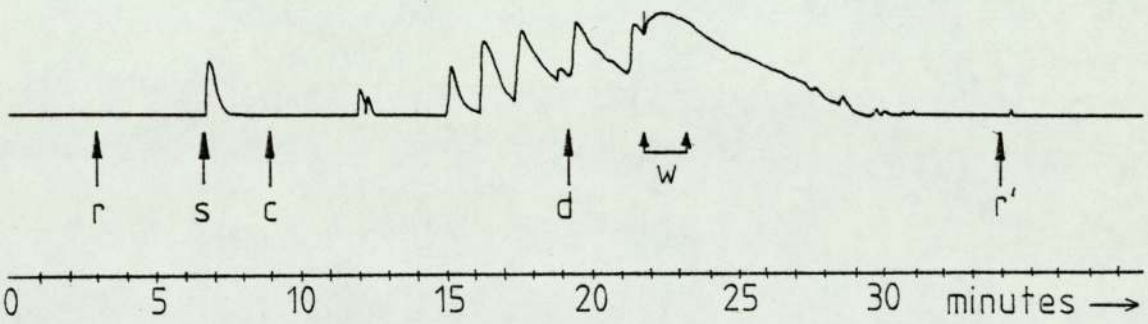
In all preparations the ability of the inhalent siphon to contract was tested before alkyllead addition, by tactile stimulation. A viable siphon always rapidly contracted and then rapidly elongated again to its original relaxed length of 3 cm. Similar responses were recorded by Akberali[108] using a 5 volt electrical stimulus.

After a steady baseline had been regained and maintained for several minutes, additions of various alkyllead compounds were made. The time

taken for responses to initial alkyllead additions varied considerably from preparation to preparation. In general the higher the final seawater concentration was above the response limit of the animal, the shorter the response time. This applies to both isolated and insitu siphons.

Both types of preparation exhibited the same range and type of responses, except that in every compound tested, insitu siphons responded at significantly lower concentrations than isolated siphons. That is, the siphonal contractions due to alkyllead were similar, differing only in the minimum concentrations of specific compounds required to elicit those responses. Typical recordings showing the inhalent siphon responses to various stimulii can be seen in Figs 4.1.All of the minimum concentrations provoking responses were determined by similar procedures.

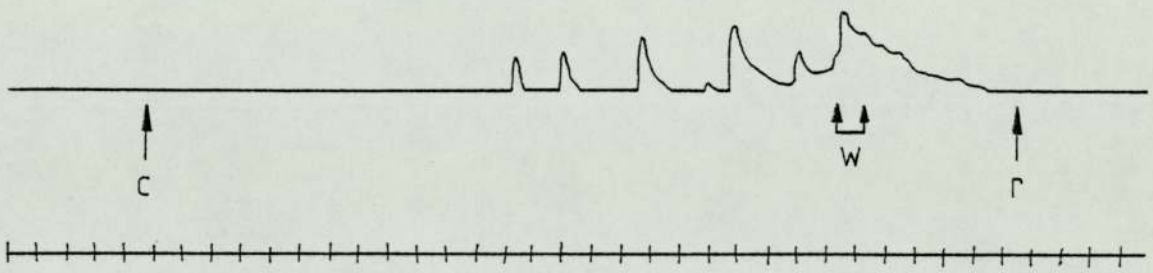
FIG 4.1a TYPICAL RESPONSE OF AN INHALENT SIPHON EXPOSED TO ALKYLLEAD



In Fig.4.1a, a typical example of insitu or isolated siphonal responses, the steady baseline reflects the inhalent siphon's relaxed, extended state (r). A tactile stimulation by pinching with forceps, produces a rapid, isotonic contraction (upward deflection), followed by a relaxation (downward deflection) (s). The siphon, in this case insitu, is then back to its steady, relaxed state. An addition of 7.5 cm^3 of a stock diethyllead chloride solution (100 mg l^{-1}) to the organ-bath at (c), to give a final Et_2Pb^{++} concentration of 5 mg l^{-1}, results in a series of contractions, increasing in magnitude, after several minutes of delay. The siphon reaches a permanently semi-contracted state (d). Washing the tissue twice with 250 cm^3 of fresh seawater and refilling the organ-bath (w), brings

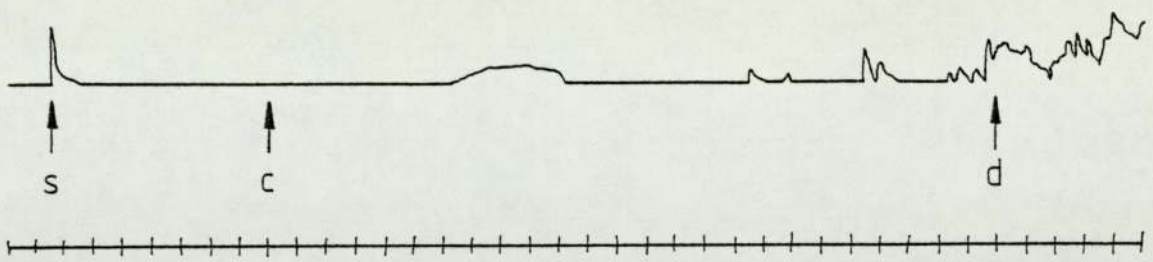
about a fairly rapid extension of the siphon to its former extended length (r). This suggests that the contractions are due to alkyllead effects, which can be washed away with fresh seawater.

FIG 4.1b ISOLATED Et_3Pb^+



In Fig.4.1b the addition of Et_3Pb^+ at (c), to a final concentration of 0.7 mg l^{-1} causes contraction in an isolated siphon after approximately 12 minutes delay. A 250 cm^3 wash (w), results in a slow relaxation of the siphon (r).

FIG 4.1c ISOLATED Me_3Pb^+

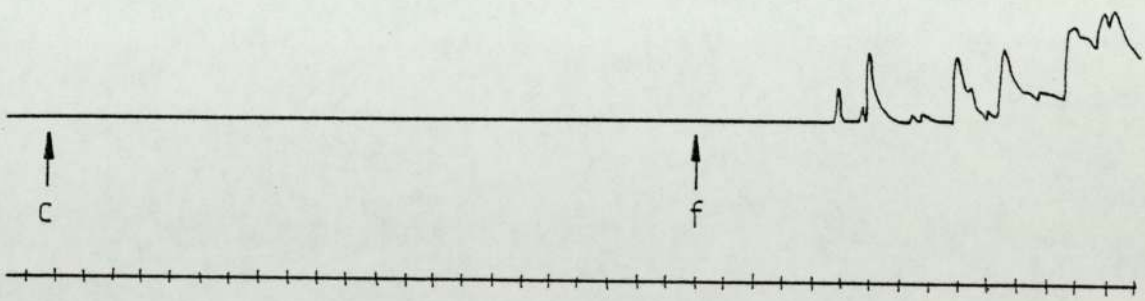


In Fig.4.1c a mechanical stimulation (s), causes a short contraction in an isolated, inhalent siphon. The siphon returns to its relaxed state and 7 minutes later 7.5cm^3 100 mg l^{-1} Me_3Pb^+ stock solution were added to give a 5.0 mg l^{-1} final concentration (c). An unusual response follows a delay of about 6 minutes. The siphon eventually reaches a state of permanent semi-contraction approximately 25 minutes after Me_3Pb^+ addition (d).

The time of response from addition of the alkyllead until the first contraction varied considerably, typically measuring between 1 or 2 and 20 minutes. The most common response for minimum concentrations producing responses was around 10 minutes. Higher, and therefore possibly more toxic concentrations usually brought about contractions after much shorter delays. Almost instantaneous responses often occurred from the highest concentrations (>5 mg l^{-1}) of the trialkyllead compounds. Such responses were not observed with the dialkyls, presumably because the concentrations required were not possible in seawater, due to solubility limitations.

Contractions occurring more than 20 minutes after alkyllead addition were not considered for minimum concentration determination because in most cases, responses fell well within this limit. The concentrations of alkyllead within the organ-bath were not always sufficient to cause a contraction. By increasing the concentration, with further additions, a response was usually observed. This method provided a rough approximation as to the minimum concentrations required for siphon withdrawal. Examples of this can be found in Figs.4.2.

FIG 4.2a INSITU. Et_3Pb^+



In Fig.4.2a, an insitu preparation, the addition of 0.15cm^3 100 mg l^{-1} Et_3PbCl stock at (c), to give an organ bath concentration of 0.1 mg l^{-1} did not provoke a response over a 22 minute period. A further 0.3 cm^3 addition (0.3 mg l^{-1}) at (f) resulted in a series of strong contractions after 4 minutes.

FIG 4.2b INSITU Me_3Pb^+

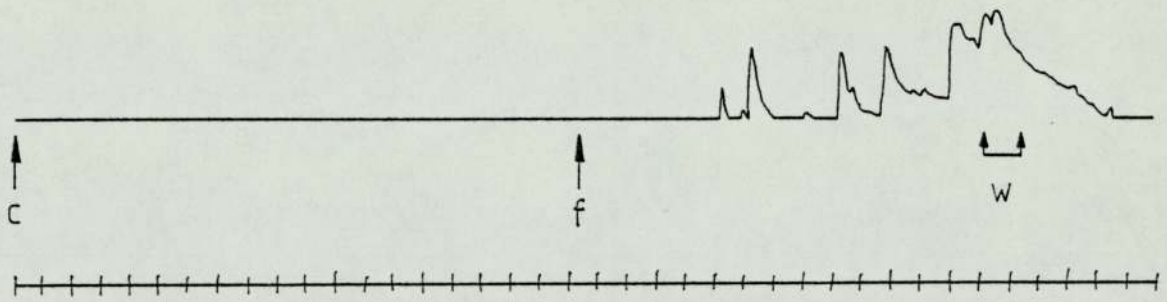
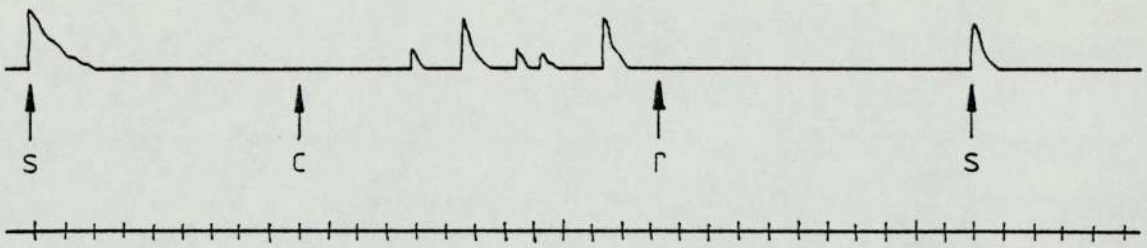


Fig.4.2b shows the lack of response to 1.0 mg l^{-1} Me_3Pb^+ added at (c). A further addition of Me_3Pb^+, (f), to give a final concentration of 2.0 mg l^{-1}, provokes contraction after a short delay. A 250 cm^3 wash with fresh seawater removes the alkyllead effects and returns the siphon to its former relaxed state.

The magnitude of contraction was highly variable (as can be seen in Figs.1 to 7), ranging from almost imperceptible tremors to complete withdrawal of the siphon in a permanently contracted state. In the majority of observations very small contractions did not occur, there being either a total absence of response or one resulting in large contractions, building up to a permanently contracted state. However, this complete withdrawal did not always remain as long as the alkyllead was present. In some preparations the initial contraction cycles lasted for a few minutes only, and then gradually fell away with the siphon returning to an extended, relaxed state (Figs.4.3.). In these cases further contractions did not occur for many minutes, or not at all, or until further additions were made to increase the alkyllead concentration. Other responses consisted of separate contractions interspaced by short relaxation periods, permanent contraction not occurring (Figs.4.4.).

FIG 4.3a INSITU Et_3Pb^+



In Fig.4.3a mechanical stimulation to an insitu inhalent siphon results in the expected short lived contraction, (s). Addition of 7.5 cm^3 100 mg l^{-1} Et_3Pb^+ solution (final concentration of 0.5 mg l^{-1}) at (c) produces a few contractions which did not progress to a permanently contracted state, but died away to a relaxed state, (r). Further contractions did not occur until mechanical stimulation at (s) again resulted in a typical response.

FIG 4.3b INSITU Bu_3Pb^+

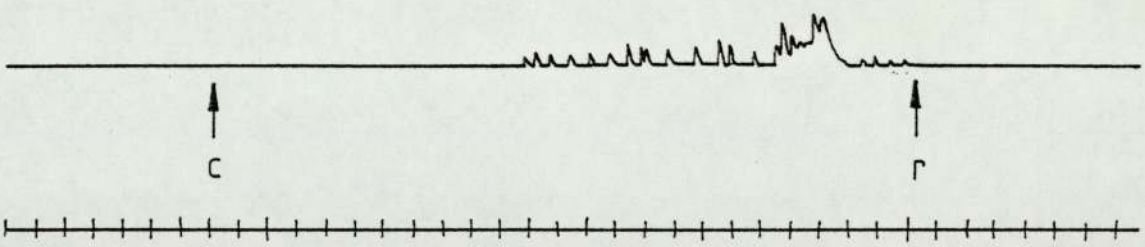
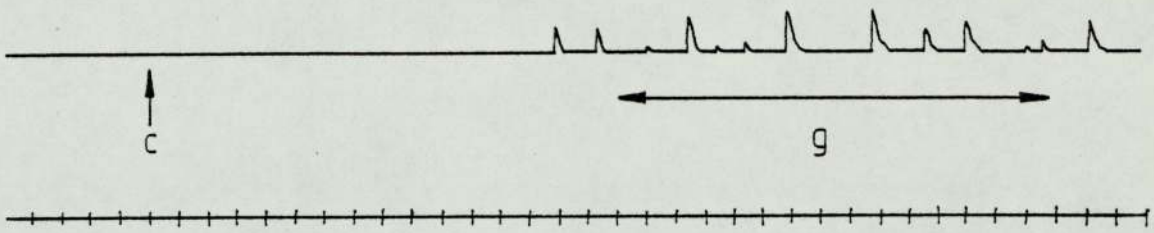


Fig.4.3b shows the response to addition of a final concentration of 0.1 mg l^{-1} Bu_3Pb^+, (c), of an insitu siphon. About 10 minutes after commencement small contractions begin, interspaced by short relaxations. A slight increase in contraction intensity after a further 9 minutes does not last and the siphon returns to its relaxed state, (r). No further contractions were observed.

FIG 4.4a INSITU Me_3Pb^+



In Fig.4.4a addition of Me_3PbCl to a final concentration of 2.0 mg l^{-1} at (c), results in a series of individual contractions, (g), after about 13 minutes exposure. The contractions were separated by relatively long relaxation periods sometimes lasting over 2 minutes.

FIG 4.4b INSITU Me_2Pb^{++}

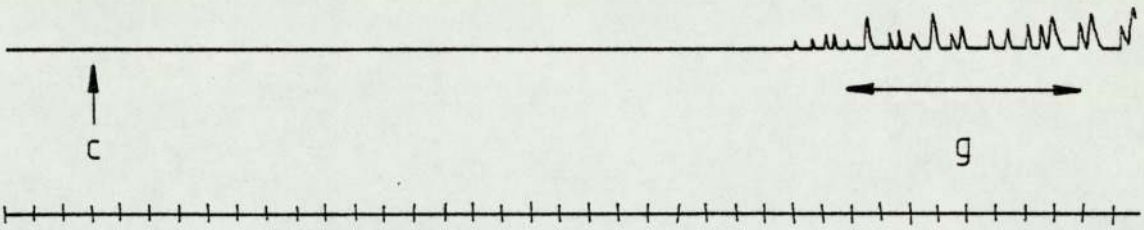


Fig.4.4b shows the separate contractions, (g), of an insitu siphon exposed to a final seawater concentration of 7.0 mg l^{-1} Me_2PbCl_2 added at (c). The exceptionally long delay of approximately 24 minutes is notable.

Because of the range of possible responses made by isolated and insitu siphons, it was difficult to decide on criteria for determining the minimum concentrations of lead alkyls which provoked responses. Presumably at some concentration, the limit of detection of the siphon would be approached, and at this point there might be a small range of

concentrations which may or may not cause a response. It was decided that the minimum concentrations should elicit at least two consecutive large contractions from the siphon, or bring about a prolonged series of individual contractions also of high magnitude. Although these criteria are purely arbitrary, they may have a significance in relation to the animal's behaviour, in that only large contractions are likely to reflect a probable closure of the valves, if the animal had been intact. It should also be noted that only a few preparations did not show either substantial contraction activity or no response.

On most occasions, after the initial contraction response to a particular alkyllead concentration, the preparation was washed and the baseline regained, as in Fig.4.1. Generally further exposure resulted in further contractions (e.g. Fig.4.5.)

FIG 4.5 INSITU Et_3Pb^+

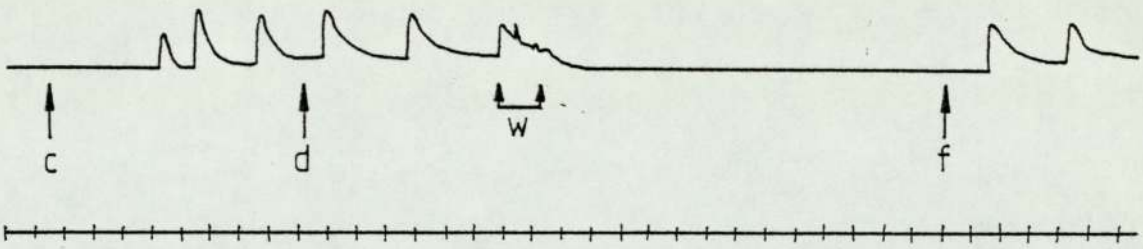
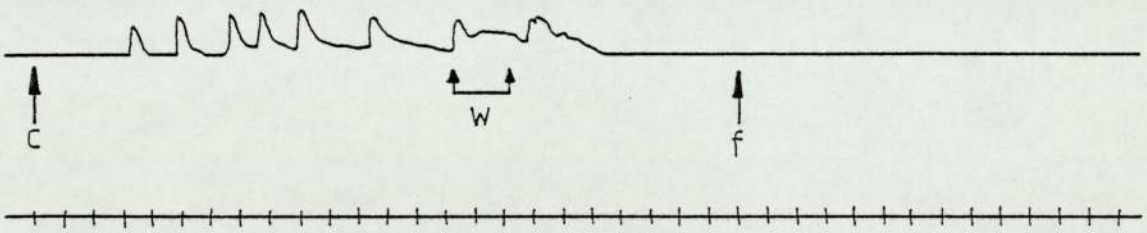


Fig.4.5. shows the typical response to alkyllead addition (0.5 mg l^{-1} Et_3Pb^+ added at (c)) resulting in permanent semi-contraction, (d). Washing with 250 cm^3 fresh seawater, (w), abated the contraction and returned the insitu siphon to a relaxed state. After about 12 minutes continuous relaxation, a further addition of 0.5 mg l^{-1} Et_3Pb^+, at (f), produced contractions after only a short delay.

However, in a few cases, further additions or repetitions of alkyllead treatments surprisingly brought about no response from the siphon. This phenomenon occurred with both tri and di- alkyl lead compounds, at

substantially different concentrations, and for both insitu and isolated siphonal preparations. In all of these preparations tactile stimulation resulted in a typical short-lived but large contraction. Such an example can be seen in Fig.4.6, where 0.2 mg l^{-1} Bu_3Pb^+ caused a typical initial response, but the same treatment after washing did not cause any contractions. Why only a few preparations exhibited this behaviour is puzzling, the majority of cases showed the expected further responses when additional alkyllead treatments were made, as seen in Fig.4.5. These more common responses give support to a faciliative or cummulative reaction to alkyllead compounds, similar to the findings of Akberali[108].

FIG 4.6 INSITU Bu_3Pb^+



In Fig.4.6. addition of 0.3 cm^3 100mg l^{-1} Bu_3PbOAc stock 'dispersion' to give a final seawater concentration of 0.2 mg l^{-1}, at (c), provokes a substantial series of contractions. Washing, (w), returns a relaxed state to the insitu siphon. A second addition of 0.2 mg l^{-1} Bu_3Pb^+ at (e) surprisingly does not cause further contraction.

Another unusual response recorded with only a few preparations, consisted of the siphon building up to a permanently semi-contracted state, after exposure to alkyllead, but several washing cycles failed to abate the contraction. Usually two 250 cm^3 washings of the organ-bath and tissue proved satisfactory in bringing about siphonal extension, but in these cases several washings did not improve the situation (Figs 4.7). This response is the complete opposite of the earlier sited examples of

permanent relaxation (Fig.4.6.) after washing, and again both insitu and isolated siphons were involved.

FIG 4.7a ISOLATED Me_3Pb^+

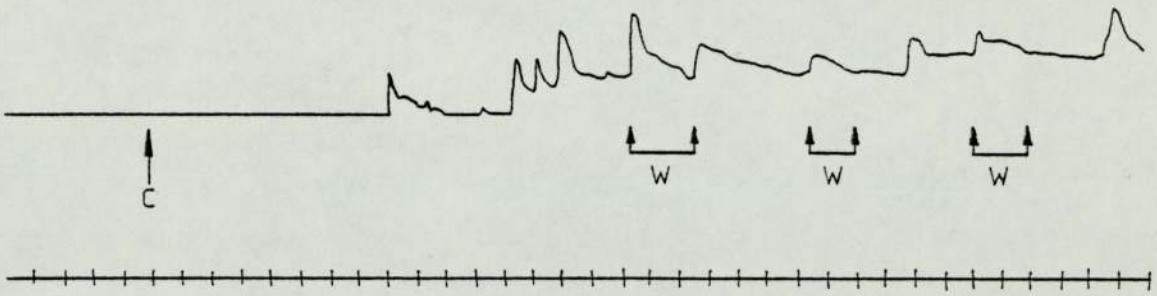
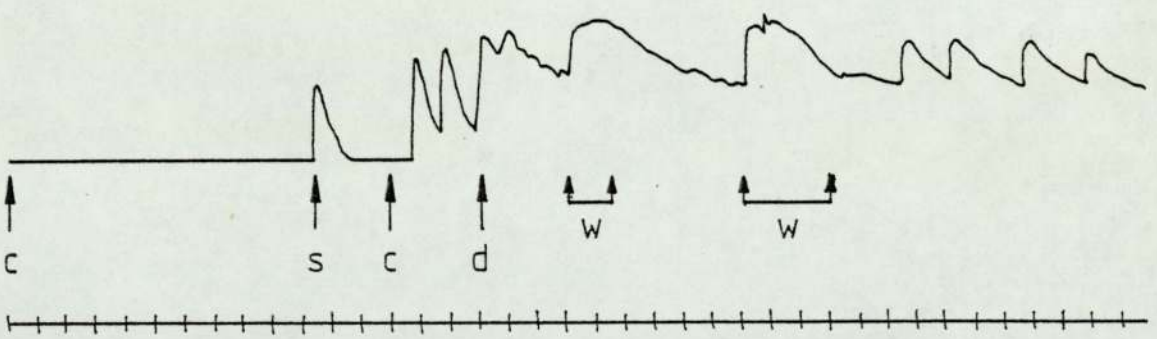


Fig.4.7a shows the response to addition of Me_3Pb^+ to a final concentration of 8.0 mg l^{-1}, at (c). When in the semi-contracted state successive washing cycles, (w), failed to reduce the intensity of contraction. Indeed complete contraction remained until the preparation was discarded.

FIG 4.7b INSITU Et_3Pb^+



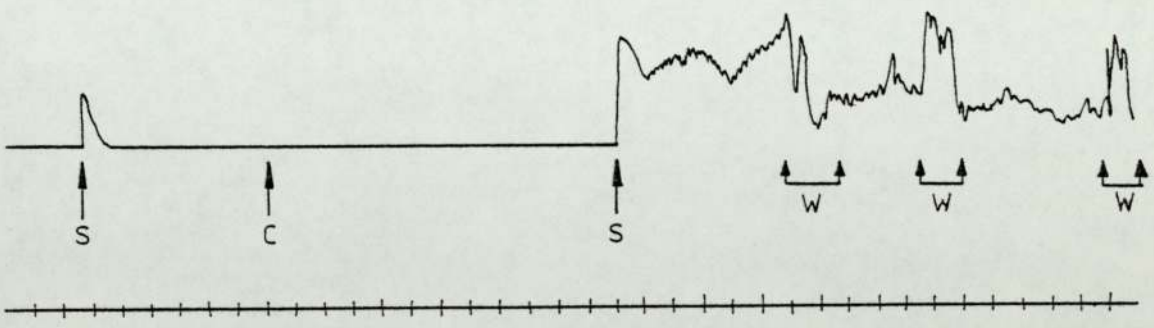
In Fig.4.7b an insitu preparation does not respond to the addition of 0.6 cm^3 100 mg l^{-1} Et_3Pb^+ stock (final concentration 0.4 mg l^{-1}) at (c). Mechanical stimulation at (s) produces a typical short but strong response. Further addition of Et_3Pb^+ at (c) to give a total concentration of 0.5 mg l^{-1} resulted in maximum contraction at (d), after a short period. Successive washes with 250 cm^3 and 500 cm^3 seawater, (w), did not abate the contractions.

The failure to obtain a relaxed, stable preparation by washing suggests

that some alkyllead may have remained in contact with the siphon even after several washes. Akberali[108] found similar difficulties when exposing isolated Scrobicularia siphons to serotonin (5-hydroxytryptamine) which induces muscle relaxation. Several washes did not eliminate the influence of the serotonin, possibly because of the high concentration used. However, in the present study there were only a few examples of failure to gain extension of the siphon, or conversely, to gain contraction. The effect of serotonin was more consistent.

Another less common and perhaps related response occurred after tri or di alkyl lead additions well above the minimum concentration required for response were added to isolated or insitu preparations. There was a complete lack of response for over 20 minutes. Then, when the siphons were pinched with forceps, a continuous series of contractions was initiated (Fig.4.8.). Thus it appeared as though the siphon required some tactile stimulation to develop the usual contraction cycle leading to permanent withdrawal. In these cases the baseline was often re-established, although several washes in addition to the initial two, were usually needed.

FIG 4.8 ISOLATED Me_3Pb^+



In Fig.4.8. contraction of the isolated siphon is expected when stimulated mechanically, (s). Addition of Me_3Pb^+ to a final concentration of 6.0 mg l^{-1} at (c) does not cause a response for 12 minutes. A second mechanical stimulation, (s), causes immediate maximum contraction which remains in spite of several washing cycles, (w).

It is difficult to explain these anomalous responses. Perhaps some physiological or biochemical process was initiated by factors not influencing the majority of preparations. Such explanations remain pure conjecture; it may be more useful and pertinent to consider the more common responses in detail.

4.2.2. Minimum Alkyllead Concentrations Causing Siphonal Contraction

If the concentration of a particular alkyllead compound causing a siphonal contraction can be taken as some indication of the toxicity of that compound, then the responses can be used to compare alkyllead toxicities. This seems to be a reasonable assumption, as increasing concentrations resulted in greater or faster responses.

TABLE 4.1 MINIMUM CONCENTRATIONS REQUIRED FOR SIPHONAL CONTRACTION IN SCROBICULARIA ($mg\,l^{-1}$)

n.r. - no response
n.d. - not done

Compound	Siphonal	Preparation
	Insitu	Isolated
$Pb(NO_3)_2$	n.r. at sat. soln.	n.r.
Me_2PbCl_2	7·0	n.d.
Et_2PbCl_2	5·0	n.d.
Me_3PbCl	2·0	5·0
Et_3PbCl	0·2	0·5
Bu_3PbOAc	0·1	n.d.
TEL	n.r.	n.d.

By considering the minimum response concentrations given in Table 4.1, it can be seen that, for the compounds tested, toxicity increases with increasing chain length, and trialkyllead compounds are more toxic than dialkyllead compounds. Tetraethyllead, however, is most likely non-toxic in its pure form.

It can be seen from the trialkyllead results that the insitu preparations were more 'sensitive', and responded to far lower concentrations than isolated siphons. For this reason it was decided to concentrate on obtaining accurate minimum response levels for the two dialkyllead compounds tested on insitu preparations. It was assumed that isolated siphonal responses would occur only at higher concentrations. Of the trialkyllead salts tested (all of which were chlorides except for tributyllead acetate), trimethyllead was the least effective requiring 2.0 mg l^{-1} of final seawater concentration to elicit the accepted response. Scrobicularia siphons were 10 times more responsive to triethyllead and about 20 times more responsive to the butyl form. These observations are supported by the toxicity experiments involving trimethyllead discussed in Chapter 3, in that triethyl was more toxic to Scrobicularia than trimethyl.

Difficulties in dissolving tributyllead acetate in distilled water and seawater were encountered so that, initially a very fine suspension was made using a sonic bath. This stock was administered to the organ-bath in the usual manner, and concentrations were calculated as if the stock were a true solution. On dilution, the suspension behaved in a similar way to the other solutions, being dispersed quickly. There was no sign of any precipitation, indeed the small amount of suspension added, may have dissolved in the greater volume of the organ-bath. After several days the compound appeared to have completely dissolved, and similar results were obtained from this solution.

Unfortunately longer chain-length compounds were not available to further test the trend of increasing toxicity with increasing chain-length. Attempts to synthesise trihexyllead chloride proved unsuccessful. Both dialkylleads were less toxic than their trialkyllead analogues the diethyl form having the greater effect. To determine further the influence of the degree of alkylation upon toxicity, TEL and inorganic lead, Pb^{++} ($Pb(NO_3)_2$), were administered to the siphonal preparations.

Inorganic lead failed to elicit any response at concentrations up to 100 mg l^{-1}. Concentrations above this were difficult to achieve due to the precipitation of lead when the freshwater stock solution was added to the seawater. However, even up to this concentration, the lack of response tended to support the hypothesis of toxicity increasing with the degree of alkylation.

Tetraethyllead has a very low solubility in water (0.3 mg l^{-1})[2,3]. To achieve higher concentrations in the test system a suspension of TEL in distilled water was made, using a sonic bath. The suspension (100 mg l^{-1} TEL in 100cm^3 H_2O) was very fine and underwent rapid dispersion on entry into the organ-bath seawater. The fine TEL droplets would presumably have come into contact with the siphon during dispersion, but no responses were observed. A lack of contraction by the siphons may have been due to insufficient TEL acting upon the tissue. In order to increase this contact, larger volumes of TEL were added to the organ-bath, dissolved in ethanol. Up to 100 µl TEL in 1cm^3 ethanol added to the seawater also failed to induce a siphonal response. In two of these experiments a small contraction was observed immediately after the TEL/ethanol mixture had been added, but this is thought to have been due to the large volume of ethanol that had been pipetted (Fig.4.9.). Similar results were recorded from ethanol only additions.

FIG 4.9 INSITU. TEL.

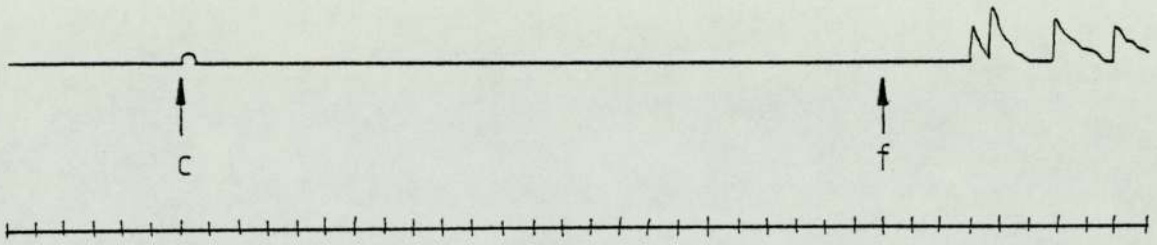


Fig.4.9. shows the lack of any significant response to addition at (c) of 100 µl of pure TEL in 1 cm^3 ethanol. An initial small 'tremor' was probably due to the large volume of ethanol added. After about 24 minutes when no response had occurred, an addition of 0.2 mg l^{-1} Bu_3Pb^+, (f), was made. This produced a series of contractions after a short delay.

It was still unclear whether the absence of response was due to TEL being non-toxic, or merely that the siphons were not being subjected to sufficient contact with the compound. Thus, ignoring total seawater concentrations but just seeking a positive response, neat droplets of TEL were administered to the siphons, insitu. A 20 µl drop delivered by automatic pipette below the surface of the seawater, and immediately above the base of the siphon, was seen to fall through the medium and land on top of the animal. The droplet struck the base of the siphon and remained in contact with it, retained in position by the shell. No response occurred. Similar droplets placed on various sections of the siphon also failed to stimulate a single contraction. As contact with TEL was observed to take place, but with no response, it appears most likely that the TEL added to the test system was non-toxic.

Earlier toxicity studies with Scrobicularia and Macoma (Chapter 3) had suggested a degree of toxicity of TEL, so the total lack of siphonal response to direct contact with TEL was unexpected. However, it remains a fact that no contraction was achieved due to TEL action. This paradox

is difficult to resolve. On the one hand direct exposure to TEL of the siphons caused no response and thereby suggests no toxicity, but on the other hand whole animals exposed to TEL in ambient seawater resulted in mortalities. Even though many or all control animals died, this last observation points to toxicity. The evidence for a low TEL toxicity is strong. In all other siphonal preparations other than TEL exposed ones, responses were observed from all compounds, albeit at varying concentrations. In other words, the test system appeared to be reliable and, of course, any particular concentration could be repeated many times on different preparations.

The evidence for TEL being toxic is not so strong, even if the numbers of mortalities from the two toxicity experiments are considered. It is more difficult to directly relate the presence of TEL in the exposure system as being the cause of these mortalities, as accumulation by the animals is unknown (due to lack of tissue analysis). The important consideration here is the unusually high control mortalities which occurred in both experiments. As discussed in Chapter 3, the condition factors of control animals fell rapidly during these experiments. If the control molluscs were heavilly stressed, then it is most likely that all of the animals used would be similarly stressed. This may have reduced their survival potential and therefore have resulted in 'higher than normal' mortalities. It is known that concentrations of Et_3Pb^+/Et_2Pb^{++} due to TEL decomposition were very low (0.1 mg l^{-1}) but toxic effects cannot be discounted because of the possibly higher susceptibility of the animals. These mitigating circumstances make it difficult to conclude TEL toxicity, especially in light of the siphonal preparation observations.

Comparing the minimum concentrations required for siphonal contractions with the concentrations used in the toxicity studies, shows that, for Scrobicularia all exposure concentrations in the former experiments

(except TEL) were near or above those resulting in siphonal responses. In other words, these concentrations were toxic to the animals. It is not surprising therefore, that during these toxicity runs, many of the animals exhibited valve closure and withdrawal of the siphons for substantial periods. However, as previously discussed in Chapter 3, no individual remained noticeably isolated from the ambient environment for very long, continuous periods. Such behaviour was only observed over several hours rather than days. In TEL experiments no long periods of valve closure were observed, in fact siphons were extended as in controls, feeding freely. These observations may be interpreted in several ways: for example, the animals may have, under natural conditions, maintained valve closure for several days, but because of the less favourable conditions of the laboratory experiment and less abundant food supplies, such long periods were not possible. Acclimation to the alkyllead containing seawater is doubtful because of the high concentrations used, and the lack of a noticeable relationship between periods of shell closure and alkyllead concentration.

4.3. Mechanisms of Toxic Action

The majority of investigations into the toxicity of alkyllead compounds have involved mammals. Most of these studies conclude that trialkyllead is more toxic than TAL, however the more closely related study involving marine organisms[51], suggests the opposite, with toxicity increasing with degree of alkylation.

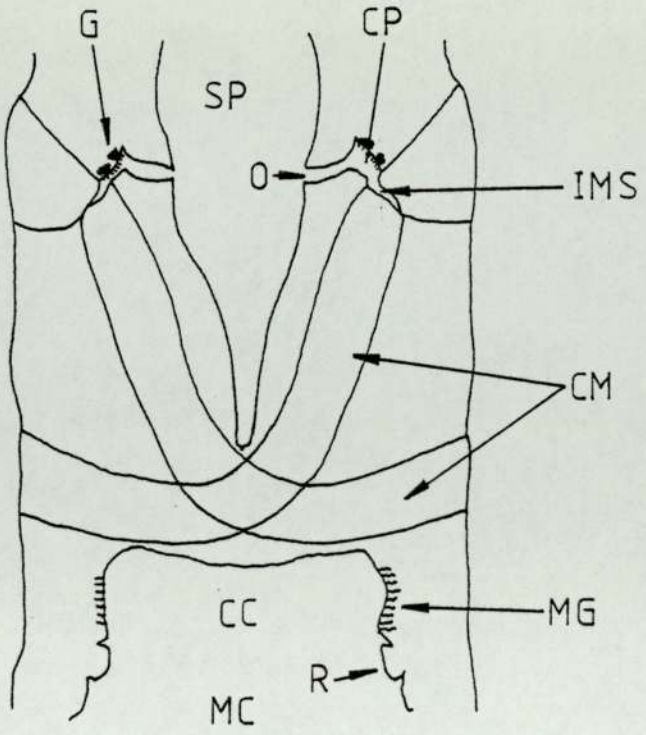
The toxcity of alkyllead compounds may be due to the lead content only, the alkyl groups merely influencing the degree of absorption. If this were so it would support the suggestion of greater TEL toxicity because of its greater lipophilic nature. Triethyllead is less lipid soluble. The present study tends to support the former work, with TEL only becoming toxic when converted to the triethyl form.

The mechanisms involved in toxin action on siphons of Scrobicularia are not fully understood[108]. The siphon's structure consists of layers of longtitudinal and radial muscle associated with beds of collagen[110]. There are six evenly spaced nerve fibres which run the length of the siphon ending in six lobes at the tip[110]. These nerves innervate the muscles to contract and generally control the mobility of the siphon.

At the base of the siphon lies the cruciform muscle (see Fig.4.10.), which is thought to take the strain of siphonal contraction and extension (111). Apart from this function, the muscle may be part of the sense organ which is present near the posterior attatchment of each muscle (see Fig.4.11.). The exact function of the sensory organ is also uncertain, but it may test water quality from the inhalent siphon and co-ordinate adductor and siphon activity, i.e. the siphons are always withdrawn before the valves are closed to avoid snipping off parts of the siphons. The presence of the intact cruciform muscle and associated sense organ in insitu preparations may explain the greater sensitivity exhibited by these siphons. Isolated siphons were excised from the organs, but still contracted on exposure to alkylleads. Therefore there must be an intrinsic mechanism of the siphon itself, capable of generating contraction.

Toxins presumably either attack the muscle directly, or affect the neuromuscular junction or nervous system. Previous mammalian studies have shown that alkyllead compounds affect the nervous system[30,37], although whether this is the only target in siphonal preparations is open to discussion. In this study it was attempted to obtain some measure of toxicity of alkyllead compounds, rather than investigating such toxic mechanisms.

FIG 4.10 POSITION OF CRUCIFORM MUSCLE WHEN SIPHONS ARE WITHDRAWN



G Ganglion below sensory epithelium of sensory pit

CP Ciliated pit (lined with sensory epithelium)

O External opening of sense organ

R Ridge bounding central channel

SP Siphonal space

IMS Intramuscular slit

CC Central channel

MG Mucous glands

MC Mantle cavity

CM Cruciform muscle

CHAPTER FIVE

TOXICITY OF ALKYLLEAD COMPOUNDS TO P.TRICORNUTUM

TOXICITY OF ALKYLLEAD COMPOUNDS TO P.TRICORNUTUM

5.1. Introduction

Our attempts to determine TEL toxicity to bivalve molluscs, both directly and indirectly, have produced some conflicting results, although this may have been due in part to the bioassay methods. It was hoped that by testing the effects of TEL on an alga by a different method, more unambiguous toxicity data would be gained.

Conflicting reports of TEL toxicity to algae are prominent in the literature. Marchetti[50] reports that the commercial antiknock compound TEL-CB, is non-toxic to Dunalliella tertiolecta below 0.1 mg Pb l^{-1}, but completely inhibits photosynthesis above 0.5 mg Pb l^{-1} after 16 hours exposure. Kozyura[112] also found TEL to be toxic to two blue-green algae Cladophera spp. and Scenedesmus spp. Maddock and Taylor[51] give a value of between 1.0 mg Pb l^{-1} and 2.0 mg Pb l^{-1} for complete photosynthetic inhibition of the marine diatom Phaeodactylum tricornutum after 6 hours exposure. In contrast, Röderer[113] concludes that TEL itself is non toxic to Poterioochromonas malhamensis, a freshwater chrysophyte, any toxic action being due to triethyllead formed by photolytic decomposition of TEL.

There are several algal assay procedures available in the literature. One of the most commonly used is the assessment of the effect on organic productivity or photosynthesis. Methods to determine other physiological or even morphological parameters are useful in toxicity assessments, but photosynthetic activity gives an accurate measure of overall growth in autotrophs, which is then easily comparable with other species. A further advantage in using photosynthetic activity of a whole culture is that it avoids repetitive and inaccurate cell counting. This would be especially applicable to Phaeodactylum which having a partially siliceous cell

wall[114], would lead to difficulty in differentiating between live and dead cells. Thus the few counts in this study, to estimate the cell density, are rough approximations only. (Cells were immobilised prior to cell counting with 0.5 cm^3 40% formaldehyde.)

To resolve the TEL toxicity problems, we decided to repeat both the Maddock and Taylor and the Rüderer work. This chapter concerns the technique used by Maddock and Taylor, and Chapter 6 that of Rüderer. Although similar work has been performed before[51], the effects of TEL-CB (commercial antiknock, including the scavengers dibromoethane and dichloroethane) had been determined, but not those of pure TEL. It was intended to extend the investigation to assess the toxicity of several other lead alkyls in order to establish the relationship, if any, between degree of alkylation/length of alkyl chain, and toxicity. By using a previously well tried technique, further comparisons between the literature and data from the present study could be made, as well as direct comparisons between different laboratories.

5.2. Alga and Medium Used

5.2.1. Alga

Because of the possibility of accidental loss of antiknock compound at sea, and to confine the investigation to a single type of medium, a marine alga was selected for testing. The species Phaeodactylum tricornutum was chosen as being convenient for several reasons: its use by Maddock and Taylor[51], thereby making direct comparisons possible, the relative ease of obtaining and maintaining cultures and finally its use as a food organism in the bivalve studies. A further advantage was that this species has been extensively studied from several view points, but especially with regard to its growth in cultivation media[115-117].

The organism was first isolated by Allen and Nelson[118] as 'Nitzschia

closterium forma minutissima', but was reclassified by Lewin[119] in 1958, forming its own sub-order. This classification is as follows:

Phylum	Chrysophyta
Class	Bacillariophyceae
Order	Bacillarioles
Sub-order	Phaeodactylineae
Family	Phaeodactylaceae
Genus	Phaeodactylum
Species	tricornutum

Phaeodactylum has been extensively cultured as a convenient food source for marine animals, as well as for studies of diatom biology. This is due to its rapid growth in relatively unspecialised media and its wide tolerance to illumination and temperature[120,121]. It has also been studied in relation to lead uptake[122]. A detailed acount of its structure and likely life history is available in the literature[114,117]. In the present study a strain from the Culture Centre for Algae and Protozoa[123] was used, this being P.tricornutum LB 1052/1.

5.2.2. Medium

The medium used was standard marine AE50 medium[123], derived from the ASP2 medium originally described by Provasoli et al[124]. The medium comprises of equal volumes of ASP2 medium and Erd Schreiberer medium, the latter being mainly a natural seawater/soil extract solution. In the present study natural seawater was substituted with artificial seawater. The ASP2 medium contains 'major' and 'minor' seawater salts, several common metal chlorides and vitamins, although the vitamins were added at a later stage.

AE50 medium was made up in 5 litre batches, by mixing the required amounts of ASP2 and Erd Schreiber media, and then autoclaving this into several 1 litre Erlenmeyer flasks. Vitamins were added after autoclaving to avoid their denaturation. Before autoclaving the AE50 medium was adjusted to pH 7.8 using concentrated HCl.

Phaeodactylum was cultured in the Erlenmeyer flasks under daylight illumination (not on windowsills) and at room temperature[125]. The medium was aerated using standard aquarium pumps (Rena 301). Sub-cultures were regularly taken to maintain the cells in a positive growth phase. The medium remained unialgal, but may have been contaminated to some extent by bacteria. Microscopic observations suggested that, when contamination had occurred, bacterial numbers were low in respect to Phaeodactylum.

5.3. Algal Assay Procedure

The procedure used was essentially the same as that used by Maddock and Taylor[51], with minor differences in conditions and substances. The method is based on the Steeman-Nielsen[126] technique for the assessment of organic productivity in the sea. The photosynthetic uptake of ^{14}C, from a known amount of labelled carbonate by a standardised number of algae, is measured over a controlled incubation period. A range of toxicant concentrations are also incorporated so that, after correction for non-photosynthetic uptake the toxicant effects on photosynthesis can be compared to control cultures with normal activity. Thus the effects due to the toxicant can be expressed as a percentage of the normal (control) activity.

The detailed procedure is as follows:
50 cm^3 AE50 medium was added to fourteen 80 cm^3 ground-glass stoppered test tubes. A precalculated volume of alkyllead stock solution was added to six of the tubes and a different volume to six others. One tube of each concentration was wrapped in aluminium foil to exclude all light. Then 2 cm^3 of a 5% sodium carbonate solution labelled with 0.1 μ Ci ^{14}C was added to each tube, followed by 2 cm^3 of a culture of Phaeodactylum (approximately 2 x 10^6 cells). The tubes were all topped up to 80 cm^3 with AE50 medium, and tightly sealed with ground-glass stoppers. Thus

there were five illuminated and one dark culture for each alkyllead concentration and one illuminated and one dark control tube. All tubes were supported by rubber holders inside a large box to exclude all external illumination. Internal illumination was by a single, fluorescent tube emitting 2000 lux to each culture. The entire experiment was placed inside an efficient fume-cupboard for safety purposes. The tubes were incubated for 6 hours with the temperature between 23°C and 25°C.

After incubation, further photosynthesis was prevented by the addition of 0.5 cm^3 40% formaldehyde solution to each tube. The cells were filtered off onto 0.45 μm cellulose nitrate membrane filters by passing the medium through the filters with 20 cm^3 syringes. The cells were then washed with 100 cm^3 of fresh, artificial seawater and were finally transferred into vials containing 10 cm^3 scintillation cocktail with the filter. The cocktail ('Cocktail T',BDH) completely dissolved both filter paper and cells in about 10 minutes. The ^{14}C activity of each vial was determined by liquid scintillation counting. Each sample was counted for 10 minutes and all samples were counted two or three times more, enabling mean values to be determined. The activity of the dark incubated tubes was subtracted from the mean of the illuminated tubes to correct for non-photosynthetic ^{14}C fixation. The activity of the alkyllead exposed cultures, which were expressed as a percentage of the normal control activity, then gives a measure of the effect of alkyllead on photosynthesis. The activities of individual samples (from several counts in the liquid scintillation counter) were used to calculate the overall mean activity of the five replicate tubes. Variation about these means is expressed as $\pm$ 1 standard deviation. The filtered 'hot' AE50 medium was acidified with concentrated nitric acid to generate $^{14}CO_2$. This was then extracted by a fume cupboard. After 24 hours, the remaining medium was discarded. All glassware, syringes, filter holders and other apparatus were

decontaminated by soaking in a strong detergent (Decon 90, BDH) for 24 hours, followed by thorough cleaning and rinsing.

Initially several replicates of control cultures were incubated to establish a mean activity for the particular number of cells present. However the difficulty in distingiushing between live and dead cells, from the cell counts, made consistent, specific start cell titers impractical. A mean activity from an accurately known cell density was, therefore, not calculated for comparative purposes. As individual cultures were likely to contain similar numbers of cells, then comparisons of dosed cultures with the respective controls only, was the most accurate method for determining the percentage activity.

5.4. Effect of Alkyllead Compounds on P. tricornutum Photosynthetic Activity

Three trialkyllead compounds, Bu_3PbOAC, Et_3PbCl, Me_3PbCl, Et_2PbCl_2 and TEL were dosed to Phaeodactylum, covering a variety of concentrations to establish their effect on photosynthesis. In addition, the influences of two environmentally abundant substances, selenium and sulphide, on alkyllead toxicity were investigated.

Concentrations discussed were established by addition to the AE50 medium of precalculated volumes of stock solutions. However, because of the radioactive contamination of the medium, instrumental analysis of concentrations within algal cells or aqueous samples were not possible. The quoted concentrations are therefore, theoretical ones.

5.4.1. Effect of Trialkyllead Chain Length

The reduction in photosynthetic activity of Phaeodactylum generally increases with increasing trialkyllead concentrations (Table 5.1 and Fig. 5.1). Above certain values further increases in concentration

FIG 5.1 EFFECT OF TRIALKYLLEAD COMPOUNDS ON THE PHOTOSYNTHETIC ACTIVITY OF P. TRICORNUTUM

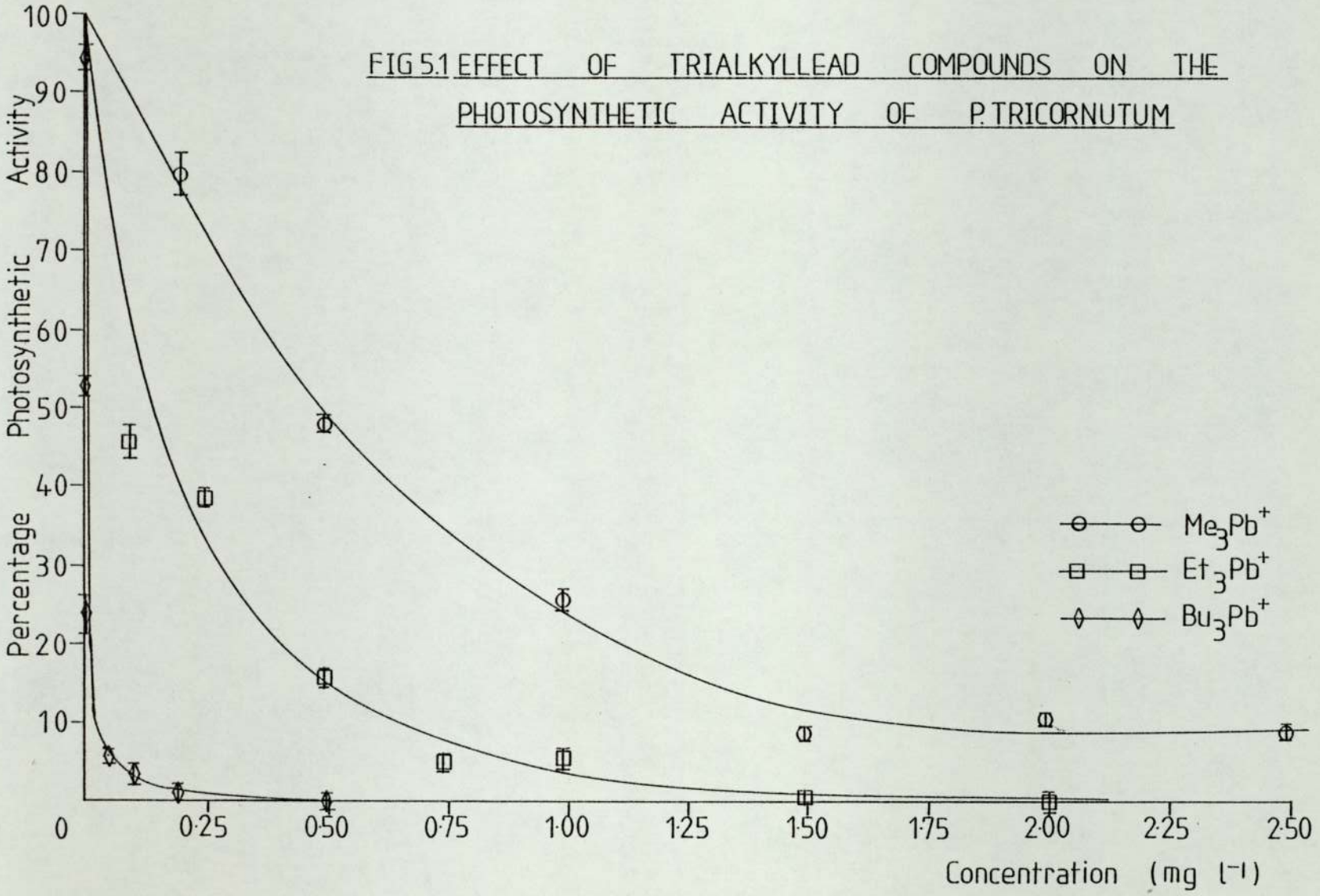


TABLE 5.1 EFFECT OF VARIOUS ALKYLLEAD COMPOUNDS ON THE PHOTOSYNTHETIC ACTIVITY OF P.TRICORNUTUM

ALKYLLEAD Conc. ($mg\ l^{-1}$)	LOG Conc.	% normal activity (± 1 S.D.)			
		Bu_3PbOAc	Et_3PbCl	Me_3PbCl	Et_2PbCl_2
4·000	0·60				19·0 ± 3·5
2·500	0·40			9·2 ± 0·6	
2·000	0·30		0·6 ± 0·6	10·3 ± 1·2	45·2 ± 3·3
1·500	0·17		0·9 ± 0·7	8·4 ± 0·4	
1·000	0·00	0·0 ± 0·0	5·9 ± 1·0	25·6 ± 2·7	56·5 ± 1·9
0·750	−0·13		4·6 ± 0·5		
0·500	−0·30	0·2 ± 0·1	16·5 ± 1·0	48·3 ± 2·7	
0·250	−0·60		40·1 ± 1·8		
0·200	−0·70	0·6 ± 0·2		79·4 ± 6·3	
0·100	−0·10	3·3 ± 0·4	46·9 ± 5·6		
0·050	−1·30	5·8 ± 0·9			
0·010	−2·00	24·0 ± 6·7			
0·005	−2·30	52·3 ± 2·7			
0·001	−3·00	95·8 ± 4·9			

result in small reductions in photosynthetic activity only. This can be more clearly seen in Fig. 5.2, where photosynthetic activity is plotted against log concentration. Here the relationship is linear over most of the concentration range, but this breaks down at the higher concentrations mentioned above.

The length of the alkyl group's straight chain appears to influence the degree of photosynthetic inhibition. For the compounds tested the longer the alkyl group, the greater the toxicity. Unfortunately attempts to synthesise trihexyllead chloride were unsuccessful (see experimental) so that further testing of this trend was not possible.

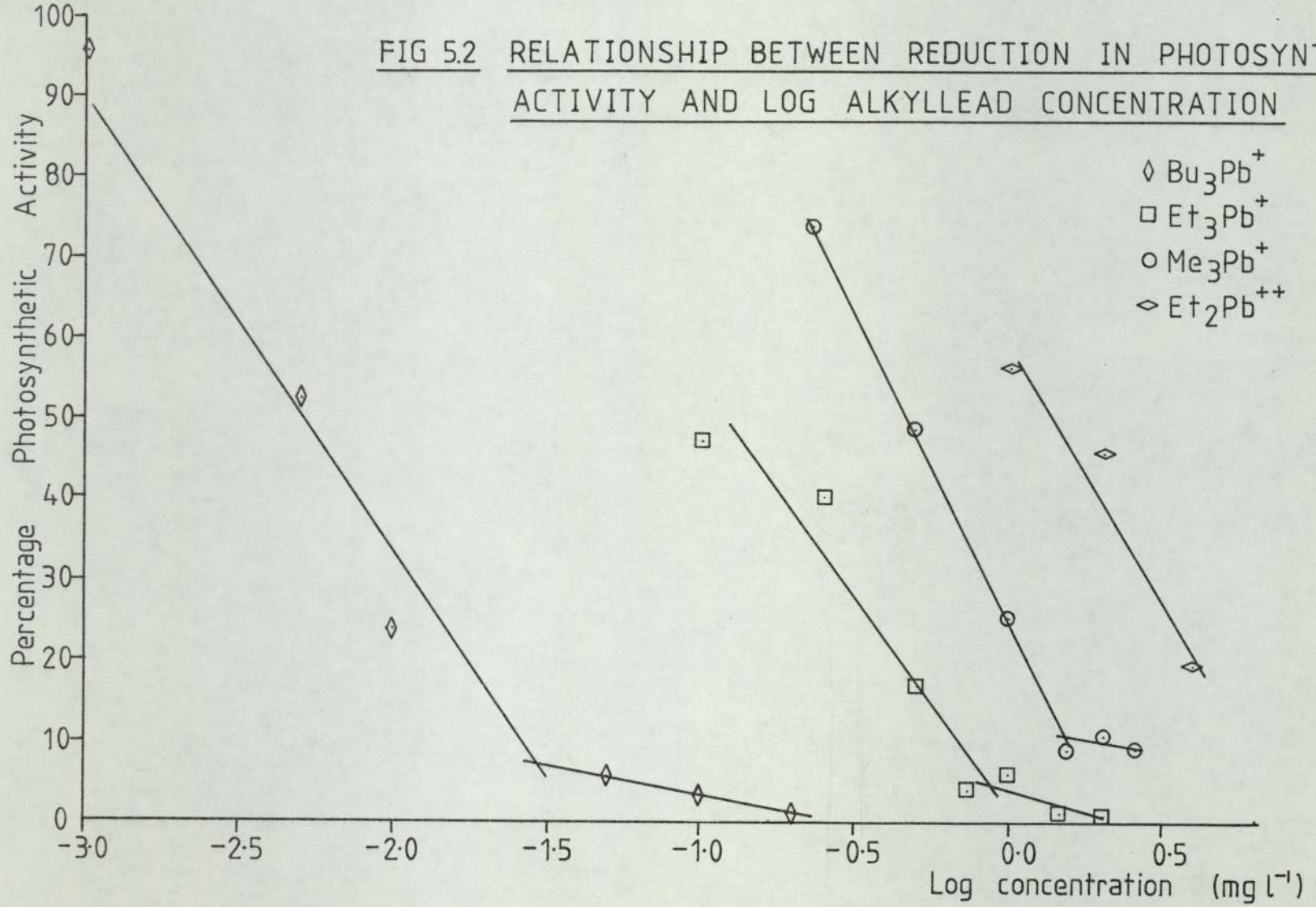
These results support the earlier work involving siphonal responses and bivalve toxicity, which also suggested increasing toxicity with increasing alkyl group size. By comparing the largest differences in photosynthetic activities caused by the two compounds at one concentration (i.e. the largest vertical distance between curves of two alkyllead compounds in Fig. 5.1), a rough estimation of the relative toxicities can be made. For example, the largest difference in photosynthetic inhibition between triethyl and trimethyl lead chlorides is at about 0.25 mg l^{-1}. At this concentration triethyllead has a 67% inhibition of photosynthesis and trimethyllead a 28% inhibition. This means that triethyllead is about 2.4 times more toxic than trimethyl. Similarly tributyllead is approximately 7.5 times more toxic than triethyl and therefore 18 times more toxic than trimethyl. Thus:

$$Bu_3Pb^+ = 7.5\ Et_3Pb^+ = 2.4\ Me_3Pb^+$$

5.4.2. Effect of Degree of Alkylation

TEL, triethyllead chloride and diethyllead dichloride were added to cultures in order to determine any relationship between photosynthetic inhibition and the number of alkyl groups attatched to the central lead

FIG 5.2 RELATIONSHIP BETWEEN REDUCTION IN PHOTOSYNTHETIC ACTIVITY AND LOG ALKYLLEAD CONCENTRATION



atom. The results are shown graphically in Fig. 5.3. In Fig. 5.3, TEL concentrations are calculated as if all of the material were in solution. This gives approximately 66 mg l^{-1} for 37% activity and 132 mg l^{-1} for 29% activity. The curve drawn is therefore a rough approximation and should be regarded with less importance than is given to the other curves. Actual TEL concentrations experienced by the algae cannot be estimated because of the presence of minute droplets of TEL in suspension, as well as TEL in solution, and the decomposition to Et_3Pb^+ further complicates the situation. The curve drawn with TEL assumed to be totally soluble is probably an underestimate of the true TEL toxicity (if TEL is toxic), as exposure from the insoluble amount actually added would be less. The effects on photosynthesis observed are, therefore, due to lower concentrations than 66 mg l^{-1} or 132 mg l^{-1}, although in reality a homogeneous exposure to algae is doubtful. As predicted from the siphonal preparation data, the triethyl compound is more toxic than the diethyl one, with TEL having the least effect on photosynthesis. Once again problems were encountered in quantifying TEL exposure, and this is discussed in 5.4.3. An approxiamte estimate of the maximum difference in degree of toxicity between di and tri ethyllead is 4, i.e. triethyllead chloride inhibits photosynthesis four times more than diethyllead dichloride.

$$Et_3Pb^+ = 4\ Et_2Pb^{++}$$

5.4.3. Effect of TEL

Once again the low solubility of TEL resulted in difficulty in establishing a known concentration. The maximum concentration, in true solution, was presumably between 0.2 mg l^{-1} and 0.3 mg l^{-1}[2,3], but the amount of TEL in suspension is unknown. As Phaeodactylum is not highly motile[114], the cells tend to sink to the bottom of the test tubes over the 6 hour incubation. There would then be the possibility of direct contact of the

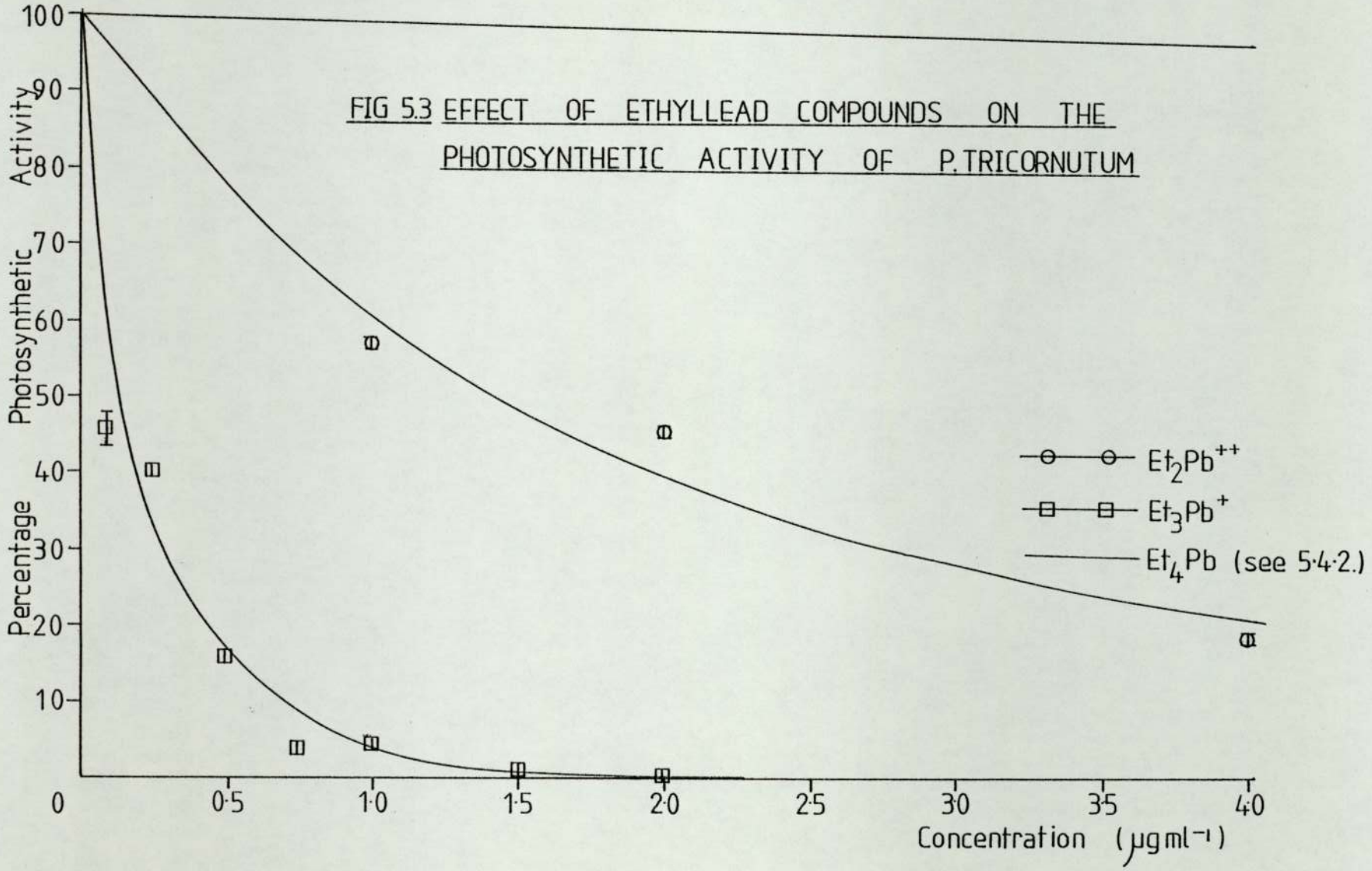


FIG 5.3 EFFECT OF ETHYLLEAD COMPOUNDS ON THE PHOTOSYNTHETIC ACTIVITY OF P. TRICORNUTUM

cells with the small droplet of TEL also at the bottom of the tube. Considering these factors, some contact between TEL and cells should take place, bringing about a suppression of photosynthetic activity, if TEL is toxic.

Initial incubations containing 62.5 µl TEL l^{-1} and 125 µl TEL l^{-1}, resulted in 36.7% and 29.2% of normal activity respectively (Table 5.3). The considerable reduction in photosynthesis could suggest that TEL is toxic, however this assumption is invalid without knowledge of the concentration of triethyllead in the system. Under strong illumination TEL would be expected to decompose rapidly, to produce the lower analogue and even in 6 hours a significant amount may have been photolysed(5).

It was not possible to analyse the 'hot' medium, so a parallel incubation was set up, under the same conditions, with the exception of omitting the ^{14}C. The analytical method used is outlined in 2.4.1.1. Analyses of the solutions were conducted every hour on two replicate tubes. The experiment was repeated later to give a total of four analyses for each hour of incubation. The results (Table 5.2) indicate an initial high concentration of TEL, probably due to droplets in suspension, but more interestingly a significant triethyllead concentration. This is surprising because at the time zero, the only triethyllead present should be that remaining after the TEL stock had been washed to remove the aqueous soluble components. Previous analyses (Chapter 3) had shown that this is a low value. Indeed, the values from AE50 medium are orders of magnitude greater than concentrations found in artificial seawater from bivalve exposures. TEL values may be the higher because of the closed system used in the algal incubations preventing any volatile losses. The smaller volume of medium would also lead to higher concentrations. One of the most important factors in high AE50 triethyllead values is strong

illumination. In bivalve studies only faint, subdued daylight reached the tanks. Contamination by TEL during the analysis is unlikely, as all TEL is extracted into the hexane layer before determinations are carried out.

Whatever the cause of the initial high value, it is important to note the general increase in concentration of the decomposition products, and the decrease in concentration of TEL. All TEL values are close to or above its true solubility, suggesting a fine suspension of TEL throughout the medium, rather than a single droplet at the bottom of the tube.

TABLE 5.2 ANALYSIS OF AE50 MEDIUM DOSED WITH 62·5µl TEL l^{-1}

Time (hrs)	Concentration (mg l^{-1})			
	TEL		Et_3Pb^+	Et_2Pb^{++}
	1	2	1	2
0	0·88	2·50	0·25	0·20
1	0·25	0·23	0·76	0·23
2	0·25	0·22	1·00	0·23
3	0·23	0·14	0·50	0·25
4	0·23	0·18	0·52	0·35
5	0·25	0·18	0·96	0·35
6	0·25	0·10	1·02	0·25

By stirring the cultures during incubation the numbers of cells in suspension would increase, as would the amount of TEL. There would then be more contact between the cells and TEL. If a difference occurred when comparing the subsequent photosynthetic activity with non-stirred, lower suspended TEL cultures, it might suggest some degree of toxicity. Thus, incubations were set up with half of the cultures stirred. A small, plastic-coated, magnetic rod was placed in each tube, which in turn was positioned above a magnetic stirring device. By this method the system remained closed, thereby preventing loss of volatile TEL.

During the 6 hour incubation period there was a small rise in ambient temperature within the box containing the tubes which was caused by the heating effect of the stirrers. By insulating the space between stirrer and test-tube with expanded polystyrene, the temperature rise in the cultures was kept down to 2°C - 3°C. All of the cultures, including the controls, underwent such temperature increases so that any changes in photosynthesis would also be included in normal activity. Control cultures were stirred and non-stirred so that any difference between both types of incubation would not be reflected in exposed culture activities. In other words, stirred cultures exposed to TEL have photosynthetic activities expressed as percentages of stirred controls, and non-stirred, exposed cultures as percentages of non-stirred controls.

The stirred cultures were, on average, 1.17 times more photosynthetically active than non-stirred 'normal' cultures (or: non-stirred = 86% stirred activity). The higher activity of stirred cultures may have been a result of the greater number of cells in suspension, preventing self shading and thereby receiving more illumination than non-stirred cells.

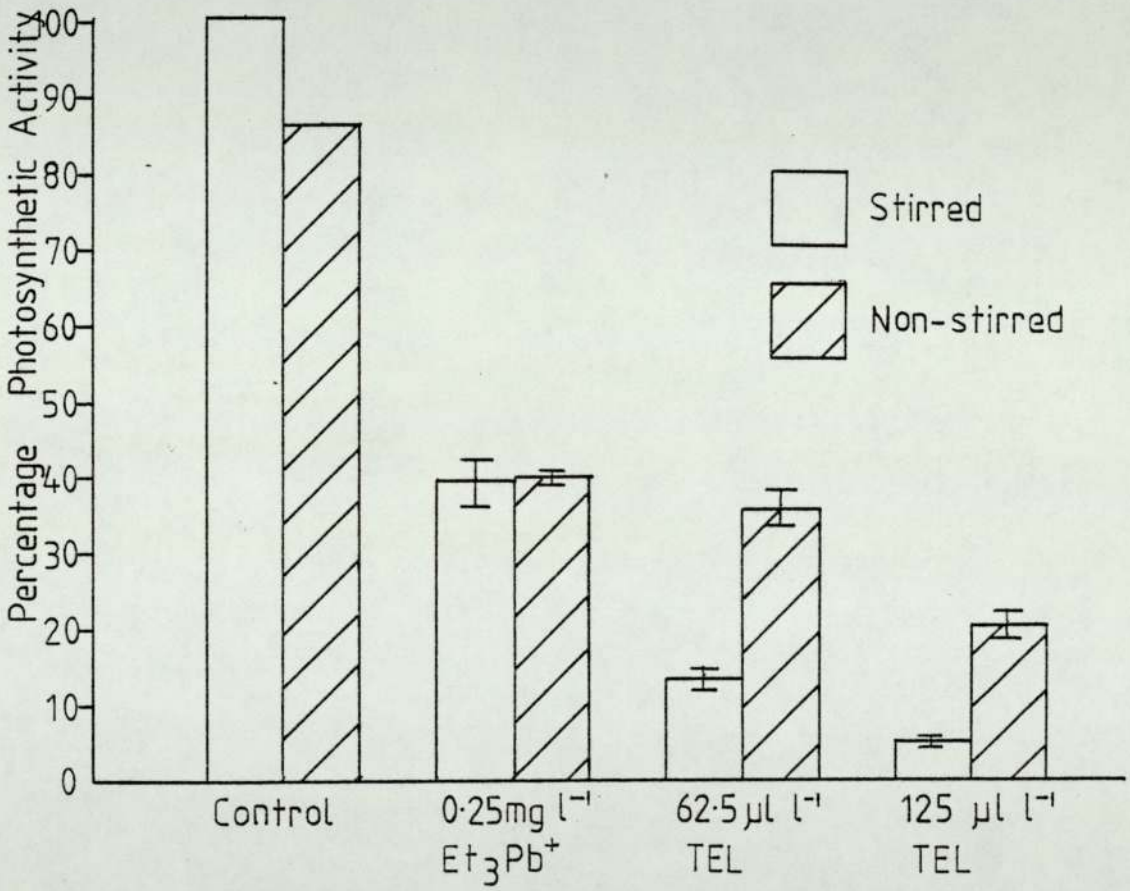
The results of TEL bioassays in Table 5.3 and Fig. 5.4 show a marked difference between stirred and non-stirred cultures, the stirred cells

TABLE 5·3 EFFECT OF STIRRING ON THE TOXICITY OF TEL AND Et_3PbCl TO PHAEODACTYLUM

± stirring	Alkyllead concentration (*)	Photosynthetic activity	
		% activity	± 1 s.d.
	TEL		
-	62·5	36·7	3·9
-	125	29·2	6·4
+	0·0	100	
-	0·0	86	
+	62·5	14·5	3·5
-	62·5	35·4	4·7
+	125	4·8	1·0
-	125	20·3	3·6
	Et_3PbCl		
+	0·25	39·4	6·2
-	0·25	39·6	2·0

(*) $\mu l\ TEL\ l^{-1}$ or $mg\ l^{-1}(Et_3Pb^+)$

FIG 5.4 EFFECT OF STIRRING ON THE TOXICITY OF TEL AND Et_3Pb^+ TO PHAEODACTYLUM



having a much lower activity. Assuming greater distribution and contact of TEL in stirred cultures, these results suggest TEL toxicity. The greater difference between stirred and non-stirred cultures with 125 µl TEL l^{-1}, than that of cultures with 62.5 µl TEL l^{-1} supports the suggestion. The higher stirred TEL dose would expose proportionally more cells as compared with the non-stirred, than the lower stirred TEL dose. Corrected photosynthetic activity for stirred and non-stirred cultures exposed to triethyllead, (Table 5.3), shows no difference in toxicity. The increased toxicity of stirred TEL cultures could, therefore, be a result of increased TEL contact with algae.

However the decomposition rate of TEL must again be considered. Stirring increases the surface area of TEL throughout the medium, and therefore a greater proportion would receive strong illumination. This might result in faster photolytic decomposition. The amount of decomposition to trialkyllead, as analysed in the initial incubations, could account for the inhibition in the stirred 62.5 µl TEL l^{-1} cultures, without having been stirred itself. Presumably the higher TEL dose would show some degree of decomposition greater than this, and with stirring might have increased further. Therefore, the suggestion that TEL is toxic is again unfounded.

5.4.3.1. Accumulation of TEL by Phaeodactylum

To establish whether contact between TEL and the algae had taken place, analyses of whole cultures exposed to TEL were undertaken. If no TEL was detected in the cells, then the toxicity observed in ^{14}C uptake studies must have been due to Et_3Pb^+. On the other hand, if TEL had accumulated, the possibility of TEL being toxic would still remain.

The experimental procedure was the same as in previously described studies, with the following exceptions:

1. The cultures were incubated for 3 days in 60 cm^3 EVT medium, containing 50 µl TEL l^{-1}.
2. The incubations and analyses were conducted in the laboratories at Associated Octel Company. The analytical procedure is described in 2.4.2. and in more detail in 6.3.1.
3. No labelled carbonate was added to the cultures which were not being stirred.
4. Estimates of growth were obtained by cell titers.

These values are likely to be inaccurate because of the slow decomposition of dead cells. Details of the cell counting method are given in Section 6.3.

The large range of TEL concentrations in aqueous samples demonstrates the difficulties involved in quantifying TEL. The values are probably all above the true solubility of TEL in seawater[3], the two values from light 1 and dark 2 cultures are obviously due to liquid TEL contamination. The data clearly show that TEL is associated with the cells, as are the decomposition products Et_3Pb^+ and Et_2Pb^{++} (Table 5.4). As only two cultures were incubated in the dark and two in the light, there are insufficient data for detailed comparisons. For example, light incubated cultures might accumulate more or less TEL than dark incubated cultures, but no trend is obvious from the results obtained. Whereas it appears from both aqueous and biological analyses that more Et_3Pb^+ is present in light incubated cultures. This would be expected, considering the photolytic nature of TEL. The concentrations of Et_2Pb^{++} in cells and medium are not so clearly divided.

It is interesting to note that concentrations of Et_3Pb^+ are approximately more than twice the respective TEL concentrations in three Phaeodactylum analyses. This may be due to TEL metabolism within the cell, or greater uptake of Et_3Pb^+ from solution.

TABLE 5.4 CONCENTRATIONS OF ALKYLLEAD IN PHAEODACTYLUM AND AE50 MEDIUM EXPOSED TO TEL IN LIGHT AND DARK

Culture conditions		Final titer(±1 S.D.) (10^6 cells/cm^3)	Alkyllead concentrations in AE50 medium (mg l^{-1})			Alkyllead concentrations in Phaeodactylum (µg Pb/10^6 cells)		
			TEL	Et_3Pb^+	Et_2Pb^{++}	TEL	Et_3Pb^+	Et_2Pb^{++}
50 µl TEL l^{-1} light	1	0·62 ± 0·11	9·30	2·08	< 0·019	0·15	0·65	0·07
	2	0·59 ± 0·15	0·91	1·64	< 0·016	0·28	0·49	< 0·01
50µl TEL l^{-1} dark	1	0·52 ± 0·08	43·0	–	–	0·09	0·21	0·01
	2	0·55 ± 0·23	0·33	0·21	< 0·002	0·31	0·19	0·07
control light		1·31 ± 0 31	< 0·001	< 0·002	< 0·001	< 0·001	< 0·001	< 0·001

It appears from the results so far gained in this thesis, that it would be extremely difficult to determine the toxicity of TEL under illuminated conditions. To reduce the interference from triethyllead breakdown products to a minimum, such experiments would be more successful in a total absence of light. This would eliminate the use of photosynthetic activity as a measure of toxicity. However, an alternative procedure capable of determining toxicity, in the absence of illumination, is discussed in Chapter 6. Similar methods to those outlined in this chapter were used by Maddock and Taylor[51] to determine TEL toxicity. In contrast to the results obtained from this study, they found TEL to be highly toxic to Phaeodactylum. However it is possible that the toxicity recorded was that of triethyllead rather than TEL[127], as they illuminated cultures at 15000 lux.

5.5. Effect of Selenium on Alkyllead Toxicity

There are many reports in the literature of the ameliorative effect of selenium on metal toxicity. In particular it has been shown to reduce the toxicity of mercury in birds[128], mammals[129], marine mammals[130] and fish[131]. Protection against cadmium, arsenic and other heavy metals has also been documented[132,133]. The mechanism of selenium action seems to be in diverting the metal to less critical tissue areas and in altering tissue concentrations[132].

It is interesting to consider whether such synergistic action might occur in unicellular Phaeodactylum exposed to alkyllead compounds. Using the ^{14}C incubation technique, cultures of the diatom were exposed to Et_3Pb^+ and Et_2Pb^{++} and various concentrations of selenite (added as Na_2SeO_3). The resulting photosynthetic activities of incubated cultures can be seen in Table 5.5. The data show large differences between 0.25 mg l^{-1} Et_3Pb^+ exposed cultures from different incubations. These values should be

TABLE 5.5 EFFECT OF SELENIUM ON Et_3PbCl AND Et_2PbCl_2 TOXICITY TO PHAEODACTYLUM

ALKYLLEAD Compound	ALKYLLEAD Concentration (mg l^{-1})	Na_2SeO_3 Concentration ($\mu g\ Se/cm^3$)	Photosynthetic Activity (%) ±1 S.D.
Et_3PbCl	0·25	0·0	53·6 ± 5·5
	0·25	10·0	38·9 ± 3·3
	0·25	0·0	43·5 ± 2·8
	0·25	1·0	46·0 ± 2·3
Et_2PbCl_2	2·00	0·0	45·2 ± 3·3
	2·00	5·0	47·2 ± 0·9
	4·00	0·0	19·0 ± 3·5
	4·00	10·0	20·0 ± 3·2
—	0·00	1·0	98·7 ± 5·3
	0·00	5·0	101·2 ± 7·1
	0·00	10·0	85·1 ± 3·3

(Individual incubations are separated by double lines)

similar as indigenous variations should be accounted for by the control tubes. Slight fluctuations in numbers of cells dispensed to each tube prior to the incubation could result in inaccurate assessments of photosynthetic activity. Small heterogeneities within the box environment might also have been present, although great care was taken to avoid this. It should be noted that variation amongst replicate tubes was small, suggesting environmental homogeneity within single incubations.

The most obvious observation is that, there is no evidence to support any significant reduction in toxcicity due to selenium protection. ^{14}C uptake does not differ from normal values when 1.0 mg Se l^{-1} or 5.0 mg Se l^{-1} are added, but 10.0 mg Se l^{-1} causes 15% inhibition. The inhibition of photosynthesis at 10.0 mg Se l^{-1} is not unexpected as selenium is well documented as being toxic itself above certain critical concentrations[132].

The absence of any ameliorative properties of selenite in all of the Et_3Pb^+ and Et_2Pb^{++} exposed cultures does not mean protection might not occur in other systems. If detoxification measures require metal diversions to non-critical tissue areas, it is difficult to imagine how this could be accomplished in a unicellular organism. Perhaps such mechanisms would operate in higher biotic systems exposed to alkyllead compounds. This aspect of alkyllead detoxification would be a most interesting area to research, but to date no such work has been found in the literature. Selenium protection in microbes, including single celled algae, has also received little attention, although selenium as an essential micronutrient is well known[132].

5.6. Effect of Sulphide (S^{2-}) on Triethyllead Chloride Toxicity

Environmental catalysts, such as sulphide, which are abundant in anoxic conditions, have been established as being able to convert trialkyllead salts to TAL and other products[24,25]. For example, the methylation

of Me_3PbCl and Et_3PbCl occurs in the following manner[5]:

$$n\ Me_3PbCl + Na_2S \rightarrow (Me_3Pb)_2S \rightarrow Me_4Pb + Me_2Pb^{++} + ---$$

$$n\ Et_3PbCl + Na_2S \rightarrow (Et_3Pb)_2S \rightarrow Et_4Pb + Et_2Pb^{++} + ---$$

Although the yields are low[25], the presence of sulphide might reduce the toxicity of triethyllead by converting it to less toxic forms, i.e. Et_2Pb^{++} and TEL. Such detoxification, as measured by an increase in photosynthetic activity, might indicate that TEL is less toxic than Et_3Pb^{+}.

The same assay system that was used with Phaeodactylum was used to measure the effects of sulphide on 0.25 mg l^{-1} Et_3PbCl containing AE50 medium. Whitmore[25] found that maximal TML production occurred when Me_3PbCl was just less than twice the molarity of Na_2S present. Therefore, initial cultures were incubated with 0.125 mg l^{-1} Na_2S. Higher concentrations were also investigated. Because sulphide solutions are readily oxidised, becoming sulphate, fresh stocks were made up for each incubation. $Na_2S.9H_2O$ was used, having a sulphide content of approximately 29%.

Again analyses of incubation medium was not possible and because of the difficulties involved in determining volatile TAL production, parallel 'cold' incubations were not conducted. The actual reactions between S^{2-} and Et_3Pb^{+} during incubation can therefore only be discussed theoretically, based on the work of similar reactions by other workers.

The results in Table 5.6 indicate some variation in activity of cultures exposed only to 0.25 mg l^{-1} Et_3Pb^{+} in different incubations. Again this is probably due to fluctuating alkyllead/sulphide doses and minor environmental changes. However this variation is covered by the standard

TABLE 5.6 EFFECT OF SULPHIDE ON Et_3PbCl TOXICITY TO PHAEODACTYLUM

Et_3Pb^+ concentration mg l^{-1}	Sulphide (S^{--}) mg l^{-1}	Photosynthetic activity %normal ± s.d.
0·25	0·00	49·7 ± 4·7
0·25	0·125	57·4 ± 5·9
0·25	0·00	41·8 ± 7·9
0·25	0·125	41·4 ± 5·9
0·25	1·00	50·2 ± 6·7
0·25	0·00	43·8 ± 2·8
0·00	10·00	70·0 ± 2·1
0·25	10·00	27·9 ± 4·8
0·25	0·00	44·6 ± 3·9
0·25	1·00	55·7 ± 8·8
0·00	1·00	101·5 ± 5·4

(Individual incubations separated by double lines)

deviations for each mean, so that in statistical terms the means could be very similar.

The initial incubations with 0.125 mg l^{-1} S^{2-} indicate a reduced Et_3Pb^+ toxicity of 7% or 8%. An increased sulphide concentration of 1.0 mg l^{-1} reduces toxicity by 9% - 13%, but at 10.0 mg l^{-1} S^{2-} photosynthetic inhibition is enhanced by about 16%. Sulphide (10.0 mg l^{-1}) exposure of Phaeodactylum, in the absence of Et_3Pb^+, resulted in a 30% inhibition in relation to normal activity. However, 1.0 mg l^{-1} showed no such reduction.

It appears therefore that S^{2-} may have converted Et_3Pb^+ to less toxic species, thereby reducing toxicity. If so, there is a reasonable possibility of TEL being less toxic than Et_3Pb^+, or even of being non toxic. On the other hand, the low concentrations (0.125 mg l^{-1}, 1.0 mg l^{-1}) may have interacted with the cells in some way to reduce the effects of Et_3Pb^+. For example, 1.0 mg l^{-1} S^{2-} may have stimulated photosynthetic activity above normal rates so that Et_3Pb^+ toxicity only appears to be lessened.

However, sulphide solutions are basic and may have increased the pH of the AE50 medium, although this was buffered to some extent. 10 mg l^{-1} S^{2-} could have altered the pH to a value away from that which is optimal for Phaeodactylum, resulting in a fall in photosynthesis. Similarly any small rise in pH from the other S^{2-} additions would be more likely to inhibit photosynthesis than to stimulate it. Nutrient limitation of sulphur compounds is most unlikely to be a cause of the data recorded.

It is therefore difficult to determine the mechanisms operating in this system from the few incubations conducted in this study. After consideration of the various factors that influence photosynthesis, the

most likely underlying mechanism in reducing Et_3Pb^+ toxicity is a direct reaction of S^{2-} with this compound, this presumably being the documented redistribution reaction to TEL and other products[5].

CHAPTER SIX

EFFECTS OF ALKYLLEAD COMPOUNDS ON POTERIOOCHROMONAS MALHAMENSIS

EFFECTS OF ALKYLLEAD COMPOUNDS ON POTERIOOCHROMONAS MALHAMENSIS

6.1. Introduction

The difficulties encountered in testing TEL toxicity, or non-toxicity as the case may be, precluded any concrete direct evidence being established. This was primarily due to the photolytic nature of the compound rapidly forming the highly toxic Et_3Pb^+ ion in all of the bioassays employed. The only way of obtaining direct toxicity results would be to excude all light from the test system, thereby minimising Et_3Pb^+ production to hopefully negligible amounts. Such a system has been used by Röderer[113,134-137] to investigate the toxicity of several alkyllead compounds including TEL, to a single celled freshwater alga.

By following Röderer's procedure and repeating some of the tests conducted by him, the conflicting literature reports of TEL toxicity might be resolved. Comparisons with the data from Phaeodactylum toxicity could also be made, to see if the freshwater system mirrors that of the marine system.

6.2. Alga and Medium Used

6.2.1. Alga

The alga used by Röderer[113] in all of his alkyllead studies was readily available in Britain from a strain maintained at the Culture Centre for Algae and Protozoa, Cambridge. Poterioochromonas malhamensis (strain 933/1a) is a primitive Chrysophycean freshwater flagellate, having a singular flagellum to provide the cell with a high degree of motility. The algae have no cell walls and consequently rapidly undergo lysis when dead[138]. The brown/yellow colouration is a result of phycochrysin pigmentation.

Classification of the Chrysophyceae is presently in a state of change, with several alternative arrangements. For this reason, placement of this

less known species shall not be attempted here.

Like many unicellular algae, generation time is short under favourable conditions, being between 5 hours and 12 hours[134], but perhaps the most relevant aspect of the organism's biology, in respect to this study is that it is not a complete autotroph. Given sufficient organic substrate and essential nutrients, the alga will grow in dark conditions as well as in illuminated medium. This facilitates the addition of pure TEL to dark cultures, preventing photolytic decomposition. Any Et_3Pb^+ formed would presumably be the result of the slower disproportionation reaction, or other chemical reactions with the medium.

6.2.2. Medium

The medium used was the inorganic EVT medium suggested by the Culture Centre for Algae and Protozoa. It consists of buffered salt solutions in distilled water which are autoclaved at 1 bar for 20 minutes, before the addition of several essential vitamins. The pH was 7.7 ± 1.0. In addition to this medium, a 0.1% glucose addition was made as an organic substrate. The majority of vitamins were added in the form of solubilised canine vitamin tablets containing homogenised liver extract. The latter also acting as an organic nutrient source.

Stock cultures of Poterioochromonas were maintained in a similar fashion to Phaeodactylum stocks, at room temperature (about 21°C), under natural daylight and in aerated Erlenmeyer flasks.

6.3. Algal Procedure

A wide variety of procedures for algal growth measurements are available and have been listed in the literature[139]. These procedures include several counting devices and dry weight, packed volume, turbidity and elemental accumulation measurements. The phenomenon of rapid cell lysis when Poterioochromonas cells die does not rule out the possibility of

cell counting to estimate growth. By applying such a method, analyses of the culture medium in which the algae have been exposed, and the cells themselves could be conducted. Although this method would be less accurate and more time consuming than the radioactive ^{14}C uptake procedure employed for Phaeodactylum, the need for analytical data (alkyllead concentrations) outweighs the disadvantages.

Röderer also estimated alkyllead toxicity by cell titers as well as determining the percentage of nucleii that were polyploid and the nuclear index[113]. In the present sudy, it was decided to count cells only, as this can be done more quickly at lower magnification than is possible when higher magnifications are required for counting nucleii. As Röderer has already established the inhibition of cytokinesis (hence polyploidy and nuclear indeces exceeding 1.0) by alkyllead action[113], the determination of these indeces was of less importance.

The bioassay system was simple. A known density of cells was incubated in $80cm^3$ EVT medium under constant illumination (2000 lux) or in complete darkness for several days. The initial cell density varied from incubation to incubation, but was generally about 0.22 million cells per cm^3 (range 0.28 to 0.18). Alkyllead compounds were added from stock solutions to replicate tubes. Equal numbers of control tubes were not dosed. Most of the apparatus used was the same as that used in the studies carried out with Phaeodactylum, including test-tubes and holders, cardboard box and lamp. Again the dark incubated tubes were covered with aluminium foil to eliminate light. In this system aeration was possible, as there was no need to observe the stringent radiological safety precautions necessary in Phaeodactylum cultures. Aeration was by a common source, feeding a single glass dropping pipette tip in each tube. The pump contained a fine filter and each pipette tip contained a 2.0 cm length of cottonwool to act as a secondary filter. Airflow to each

culture was measured at 11 l hr^{-1} ± 2 l hr^{-1} S.D. As previously discussed, aeration causes a 'washing' action removing volatile TALs from culture tubes. To reduce the speed of this process the pipette tips were placed half-way down the culture tubes, so as not to disturb the small TAL reservoir at the bottom of dosed tubes. Although TAL was lost, and the volume of TAL drop decreased, at the end of most incubations some neat TAL reservoir remained. TML is more volatile and more soluble than TEL[12] and consequently was washed out of the medium more readily. However, only in one TML incubation did complete loss of a reservoir take place. This is discussed in Section 6.6.2. The temperature of cultures remained quite constant because of the enclosed environment and heat given off by the lamp. Temperatures ranged from 24°C to 28°C, but were normally 26°C.

Cell counts were made every 24 hours by sampling each culture six times. Immediately before a sample was withdrawn, the culture was shaken vigorously to obtain an even suspension. Samples were taken using automatic pipettes, and each measured 50 µl. Cells wereimmobilised immediately prior to counting with 1 µl 40% formaldehyde solution. Although this resulted in the death of the algae, each titer only took a few minutes and was completed before lysis of cells became significant.

Cells were dispensed into a Neubauer haemacytometer, enabling consistent and accurate volumes of medium to be counted. The haemacytometer was placed upon the stage of an optical microscope for cell counting under x 100 magnification. The addition of 1 µl formaldehyde was not considered in subsequent calculations, as this error would be constant and would produce only minor alterations in cell density. Cell titers are expressed as millions of cells per cm^3. Titers can only be an approximation of the true cell densities, as only small volumes of each culture ($0.6mm^3$)

were counted. However, this method of estimating the growth of Poterioochromonas is convenient as the whole culture does not have to be included.

The highly variable cell counts, as indicated by the standard deviations of means, are the result of heterogeneity in cell dispersion within the medium. Even though the cultures were shaken immediately before aliquots were taken for counting, a complete and equal separation of individual cells was not obtained. Aggregations of cells still occurred although the association did not appear to be very strong. Titers were based on free cells only, aggregations were ignored. The cultures remained unialgal although some bacterial contamination was observed. Few bacteria were seen during counting and these were easily distinguished from Poterioochromonas cells by their lack of colour and smaller size. All cultures were similarly affected.

6.3.1. Analysis of Algal Cells

Accumulation of alkyllead compounds by the cells was analysed at the Associated Octel Company using the analytical procedure outlined in 2.4.2, usually after 3 days incubation. However, before the cells were ready for analysis they had to be extracted from the medium, a task which presented several problems. Cultures were centrifuged to obtain a pellet of cells at the bottom of the tube. The supernatant medium was then discarded or analysed for its lead content. The pellet could be resuspended in fresh EVT medium and centrifuged again in order to reduce any residual alkyllead contamination. Investigations revealed that three such 'washes' were required to remove all traces of non-cellular associated alkyllead. This is shown in Table 6.1.

Cultures of Poterioochromonas were incubated in 1.3 mg l^{-1} Et_3Pb^+ containing medium for three days. After incubation, the cells were

TABLE 6.1 ANALYSES OF 'WASHED' EVT MEDIUM AFTER EXTRACTION OF NON-CELLULAR ASSOCIATED ALKYLLEAD.

a, b, c — 1·3 mg l^{-1} Et_3Pb^+ exposure

d, e — 62·5 µl TEL l^{-1} exposure

Wash number	Alkyllead concentration ($mg\,l^{-1}$)						
	Et_3Pb^+/Et_2Pb^{++}			TEL	Et_3Pb^+ Et_2Pb^{++}	TEL	Et_3Pb^+ Et_2Pb^{++}
	a	b	c	d	d	e	e
1	0·020	0·030	0·035	0·010	0·023	<0·003	0·015
2	0·010	0·025	—	<0·003	0·008	0·005	<0·003
3	<0·003	<0·003	<0·003	<0·003	<0·003	<0·003	0·005
4	<0·003	<0·003	<0·003	<0·003	<0·003	<0·003	<0 003

sedimented by centrifugation and washed in fresh EVT medium. This was repeated three more times. Analysis of the washings (EVT supernatant) show an acceptable level of alkyllead is reached after three washings. This precedure was adopted for all algal extractions. Depuration of alkyllead from the algae may have been proceeding during washes but as each resuspension lasted a few minutes only, negligible amounts would have been lost.

The problem of obtaining algal material by centrifugation was when TEL exposed cultures were analysed. TAL has a greater density than that of water and is consequently sedimented out with the algae. Washing with an aqueous medium would not remove this TAL, which might amount to several

microlitres. Washing in an organic solvent such as hexane was not possible as the cells are adversely affected. The removal of excess TAL was, therefore, an extremely difficult objective. Total separation was not achieved, but the greater part was removed in the following manner:

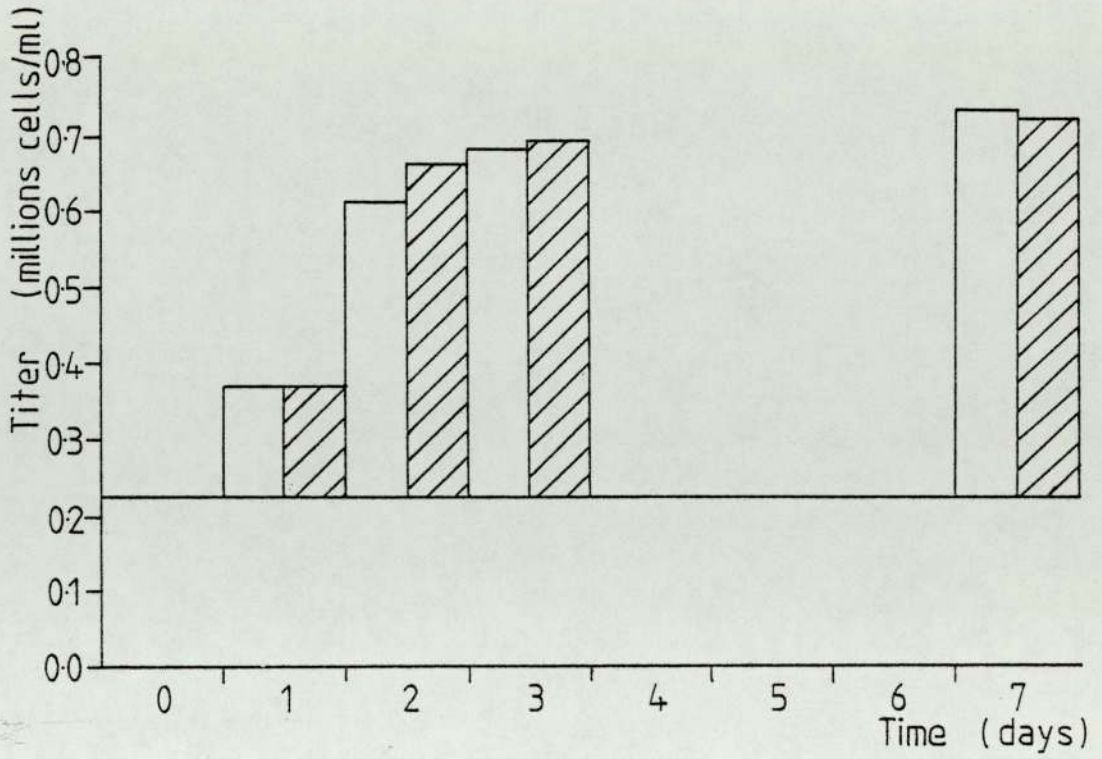
Before centrifugation all droplets of TAL at the bottom of the tube were collected by using an automatic pipette. This was done when the cells were well suspended so that minimal algal losses were encountered. The cells were then sedimented into a pellet and washed three times to remove any R_3Pb^+ from solution. After this procedure, no TAL could be seen when the pellets were resuspended. Analyses of washing medium showed removal of trialkyllead and TEL in solution. However, some residual TEL may still have been associated with algal pellets. The final pellet was placed in an efficient fume cupboard, shielded from strong light and then left for 10 minutes at room temperature. This was to allow any remaining TAL to volatilize and leave the cells. The sample was then treated in the normal way for biological analysis. Analytical results for TAL must, therefore, be treated with caution as they are likely to be overestimates of TAL actually bound or absorbed by the cells.

6.4. Growth of P. malhamensis

Under the conditions in which Poterioochromonas were maintained, growth exhibited a typical pattern, both light and dark cultures being very similar (Fig.6.1). Initial rapid growth occurred during the first few days, but by the third day had slowed, most probably due to such limiting factors as nutrients. After this, culture growth was slow, increasing only slightly above the cell density observed at 3 days. This suggests that the culture had reached a stationary phase where growth was just greater than death.

Most cultures showed this trend, which was also observed by Röderer[138].

FIG 6.1 TYPICAL GROWTH OF P.MALHAMENSIS IN MODIFIED E.V.T. MEDIUM IN LIGHT AND DARK



Hence, cultures were counted only for the first 3 days of incubation and on the 7th day, to record any deviation from this pattern caused by alkyllead toxicity. Some control cultures did not exhibit rapid exponential growth over the first three days of incubation. Indeed a few did not show increases in cell titer much above the starting density. Röderer[113] observed growth increases of two orders of magnitude over three days, but in the present study a ten fold increase in density was as much as any culture achieved. This subdued growth may have been the result of nutrient limitations, although this would more probably result in an earlier stationary phase. The temperature of incubated cultures was negligibly different to those from Röderer's cultures, eliminating this as a possible cause. The pH of Röderer's medium is not known, but is not likely to be very different from the values in this study because of the similarities in constituents. The strain used was also that used by Röderer, in spite of having been obtained from different sources.

The major difference between the two systems was in the light intensity reaching the cultures. In the present study intensity was $^{1}/_{9}$ of that used by Röderer. This might explain the lower growth rates obtained in illuminated cultures, although Röderer states that control culture growth was inhibited upto 65% compared to dark grown cultures, by the 18000 lux used by him[113]. This does not explain discrepancies between dark grown cultures. It is difficult therefore to find the reason for the discrepancy in growth rates. The low increases recorded in many of the control cultures are disturbing, but not completely unacceptable. As long as alkyllead exposed cultures are compared to the corresponding control cultures the effects of alkyllead toxicity should be discernible. However, when control cultures did not show significant increases in density, the experiment was repeated.

The toxicity of Et_3PbCl, Me_3PbCl, TEL and TML was investigated using Poterioochromonas. TML in its pure form was obtained and used in the laboratories of the Associated Octel Company. Small volumes only were available for use in accumulation studies. Cell titers were obtained over the three day periods of these incubations.

6.5. Toxicity of Et_3PbCl and Me_3PbCl

The concentration of these compounds dosed to the Poterioochromonas cultures was chosen by considering the probable amounts produced in three days of dark decomposition of TAL. Estimates from TEL incubations suggested 0.1 mg l^{-1} Et_3Pb^+, and from TML incubations 0.1 - 0.3 mg l^{-1}. By assessing the toxicity of these concentrations the possible toxic effects of TEL and TML could more easily be determined. If the toxicity from Et_3Pb^+/Me_3Pb^+ incubations was similar to that from TEL/TML incubations, the liklihood of TAL being non-toxic would be great, with the trialkyl compounds acting as sole toxins in TAL incubations. (Estimates of Et_3Pb^+ concentrations after three days photolytic

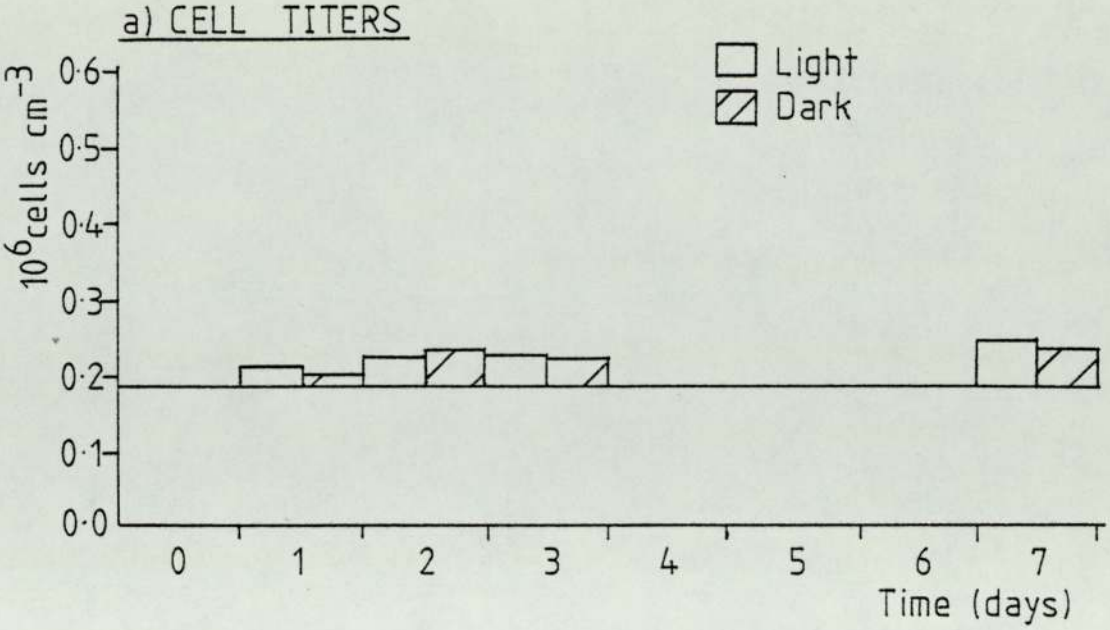
decomposition were about 1.0 mg l^{-1}. This concentration proved highly toxic to Poterioochromonas, and insufficient cells were left for analysis.)

Concentrations of 0.1 mg l^{-1} for both trialkyllead salts were used to enable comparisons of the relative toxicities. Again additions were from standard stock solutions of known concentrations. Unlike the radioactive AE50 medium used in Phaeodactylum incubations, analysis of EVT medium was practicable. Therefore most of the true exposure concentrations are known from analyses of medium at the end of the incubation periods.

As previously discussed, control cultures showed an unexpected range of growth rates from different incubations, and even titers from the same incubation were widely distributed. This variation is important when comparing the relative toxicities of different compounds. A convenient method of expressing toxicity and allowing for control variation is to calculate the percentage growth of control cultures over dosed cultures. This is a similar measure to that used in the Phaeodactylum work, where toxicity was expressed as a percentage of normal photosynthetic activity. Where control titers from different experiments are similar, a more direct comparison of toxicity can be made. However no two incubations produced values that were significantly alike, so that any such comparison would only give a very approximate value of relative toxicity.

These considerations are clearly illustrated in Figs. 6.2, 6.3 and 6.4. Both 0.1 mg l^{-1} Et_3PbCl Experiments 1 and 2 (Figs 6.4a and 6.2a respectively) suggest that Et_3PbCl is more toxic than Me_3PbCl when cell titers are compared to 0.1 mg l^{-1} Me_3PbCl titers (Fig. 6.3a), because both Et_3PbCl exposed incubations have lower growth rates than the Me_3PbCl exposed cultures. However, if the toxicity is expressed as a percentage of normal (control) growth, only Et_3PbCl Experiment 2 (Fig. 6.2b) suggests a higher toxicity than Me_3PbCl. The other experiment (Fig. 6.4b) shows similar

FIG 6.2 EFFECT OF 0·1 mg l^{-1} Et_3PbCl ON P. MALHAMENSIS



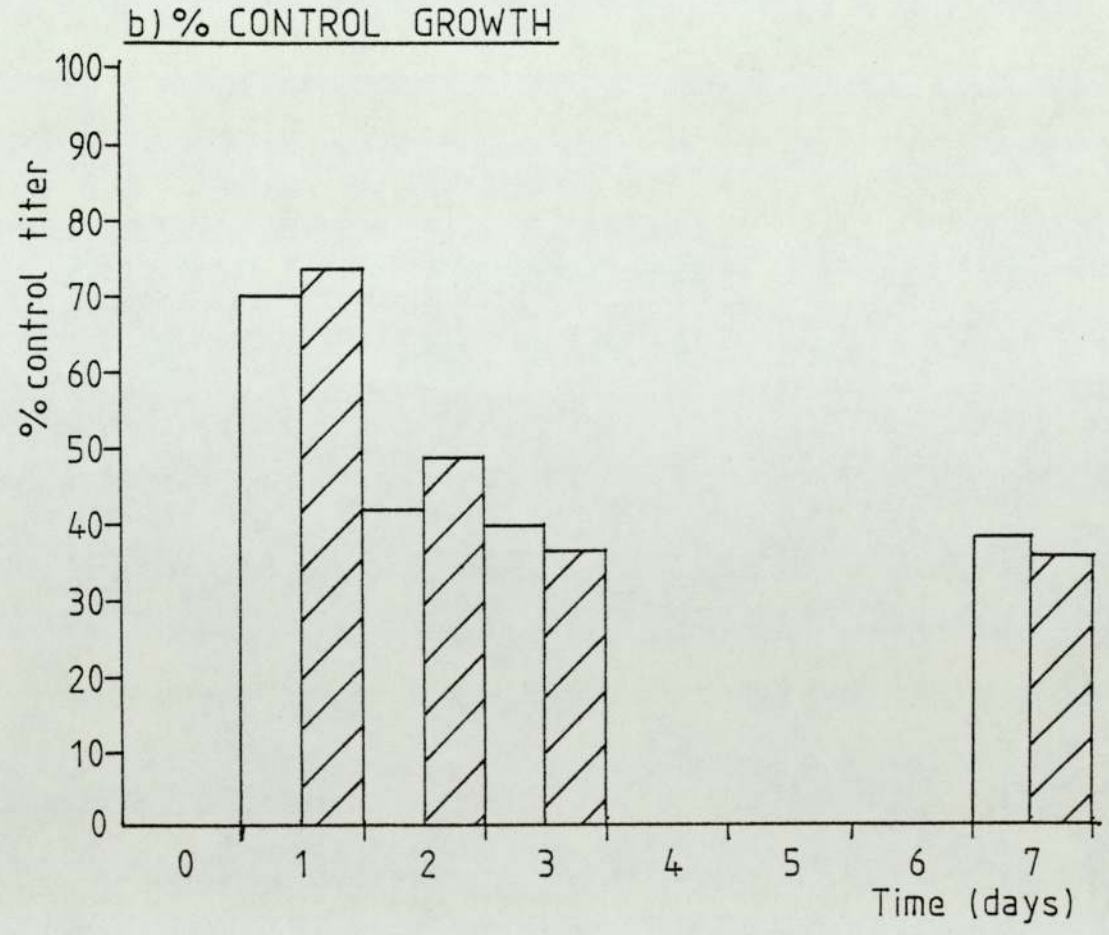
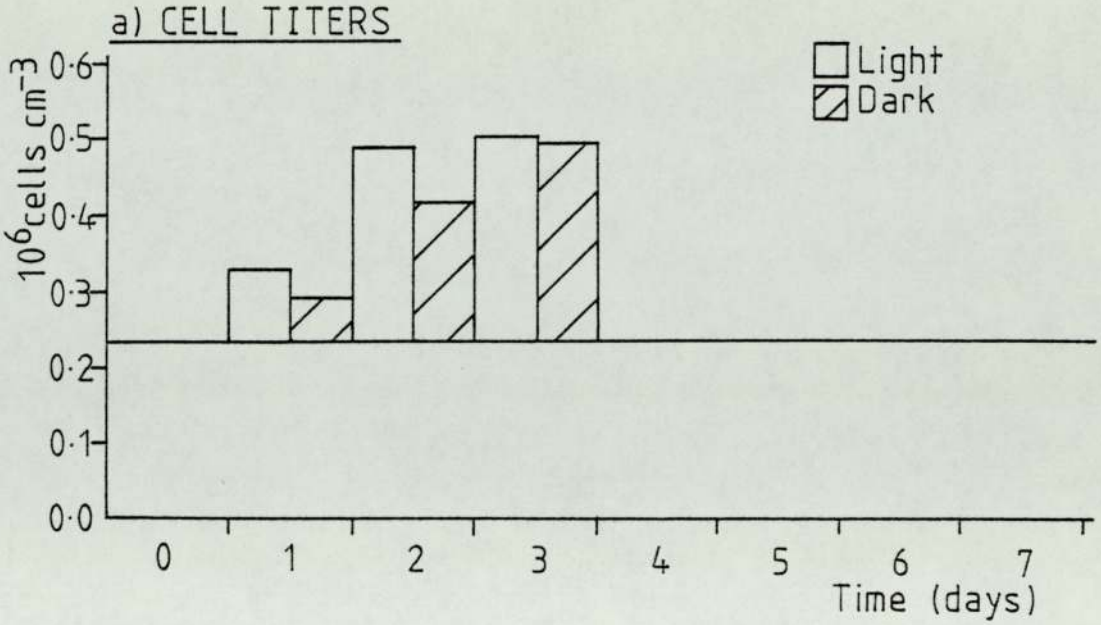


FIG 6.3 EFFECT OF 0·1 mg l^{-1} Me_3PbCl ON P.MALHAMENSIS



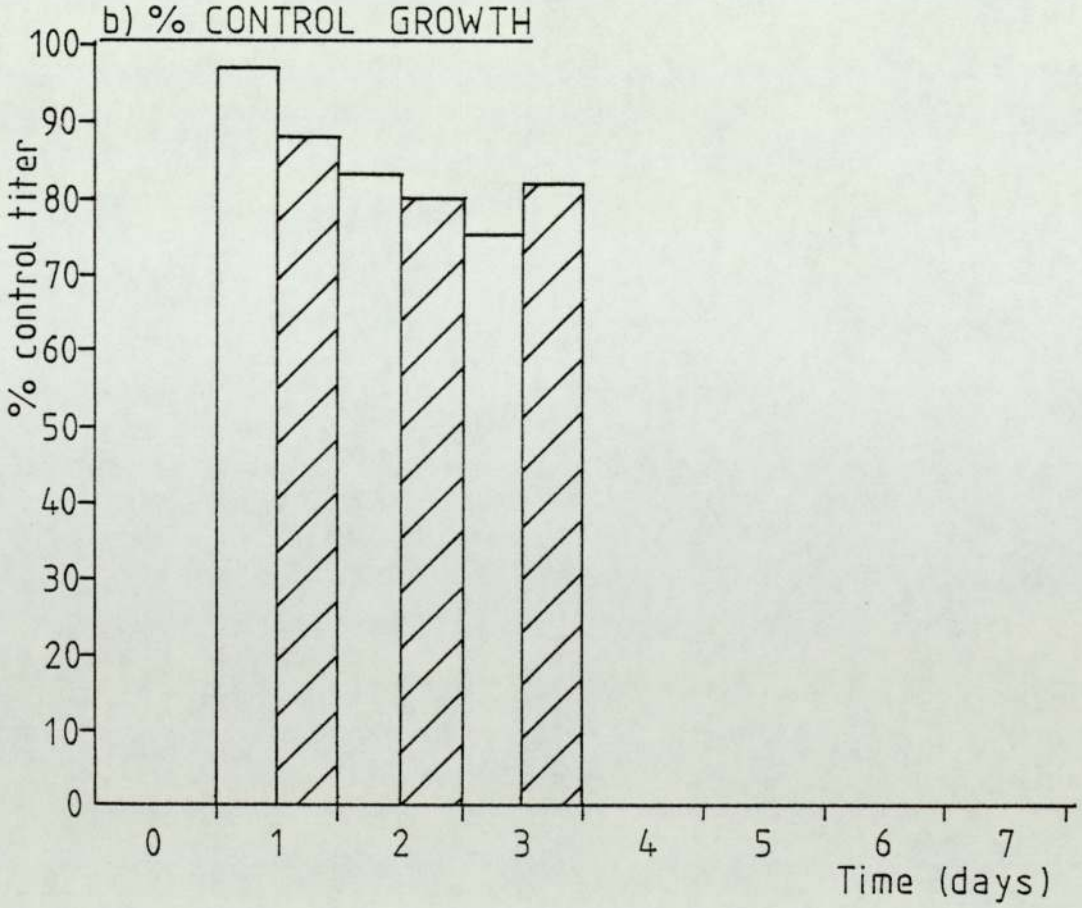
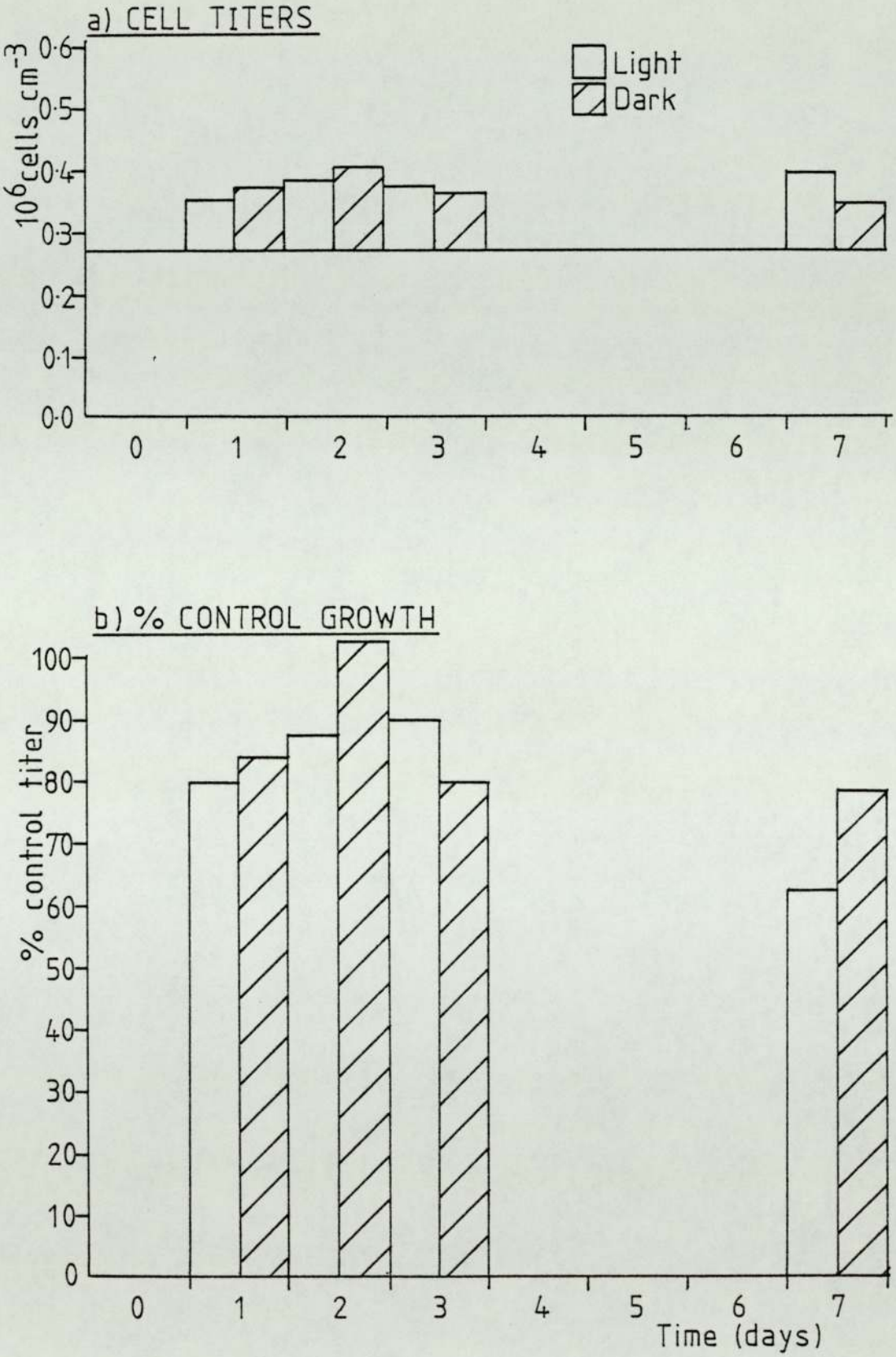


FIG 6.4 EFFECT OF 0·1 mg l^{-1} Et_3PbCl ON P. MALHAMENSIS



growth rate decreases to the Me_3PbCl incubation (Fig. 6.3b). This is because control growth in Et_3PbCl Experiment 1 is much lower than in Experiment 2, and control growth in the Me_3PbCl exposed experiment is similar to that in Experiment 2.

Although comparing percentage growth values should account for variations between incubations, inaccuracies might also be created by such parameters. Calculations of percentage normal growth can only be precise indications of toxicity if growth rate remains constant in all cultures and at all cell densities. However it is doubtful that this is the case. The earlier described growth characteristics of cultures maintained for a 7 day period obviously show a fall in rate after the first few days. Such a fall in growth rate might not occur in cultures of lower density, resulting from alkyllead exposure after similar incubation periods. This would be because fewer cells presumably utilise resources in the medium at a lower rate and they would therefore last longer.

Comparisons between different incubations should consequently be made with percentage growth values calculated from periods when the control cultures were still in the rapid growth phase, i.e. upto day 3 of the incubation period.

The incubations were both illuminated and kept in the dark to assess the effects of light upon toxicity. The trialkyllead salts undergo photolytic decomposition[5] although at a much slower rate than TAL. During either the 3 day or 7 day incubations, sufficient photolysis might occur to produce a reduction in toxicity. (Presumably the decomposition would lead to dialkylleads which are generally accepted as being less toxic than trialkyllead compounds.) Within the limits of variation from titers, there is no evidence for such a light induced detoxification of either compound. There are significant differences between light and dark cultures, but light titers are not consistently greater or smaller than

dark titers.

6.6. Toxicity of TML and TEL

The same incubation conditions as those used to assess trialkyllead toxicity were applied to the two TAL compounds. With TAL more emphasis was placed upon the importance of light and dark incubated cultures in determining toxicity. All previous attempts to quantify TAL toxicity have proved inconclusive because of the unavoidable photolysis reaction producing Et_3Pb^+. By excluding the light and analysing the various alkyllead components in the medium it was possible to exclude the effects of TAL decomposition. The data, expressed as for R_3PbCl experiments, show large, significant differences between light and dark cultures, the dark grown cells being much less affected by TAL than the light grown cells.

6.6.1. TEL

Two experiments exposing Poterioochromonas to 62.5 µl TEL l^{-1} produced very similar effects (Figs. 6.5 and 6.6). Titers (Figs. 6.5a and 6.6a) initially show a small increase over the start titer but after 48 hours exposure, the dark cultures have increased by more than twice that achieved by illuminated cultures. After 72 hours the dark cultures slow down, although still increasing overall, whereas light cultures undergo a titer decrease. After 7 days the dark cultures have fallen in cell density but this is slight in comparison to the illuminated cells which have fallen well below the original titer. Percentage normal growth values also give the same trend of very poor growth in light grown cells, with dark grown cells remaining closer to normal growth.

These results can only be explained satisfactorily by considering the changes in alkyllead compounds in the medium itself. Some data are available for concentrations throughout the 7 day incubation, but as

most cultures were analysed after 3 days incubation, EVT concentrations at this time are well replicated. The values for all other times, other than initial (time 0) concentrations, are singletons. Initial concentrations were analysed from 80cm^3 volumes of EVT medium + 5 µl TEL (62.5 µl TEL l^{-1}) after a few minutes dark incubation. The medium was sampled at about 4 cm below the surface, after it had been swirled to stir the contents. Shaking was avoided to prevent TEL gathering at the surface. (All cultures were similarly treated on TEL addition.)

The analyses were performed by ASV and GLC-MS for ionic and TEL compounds respectively, enabling determination of both Et_3Pb^+ and Et_2Pb^{++} decomposition products. The values obtained after 3 days incubation vary greatly, especially TEL concentrations (Table 6.2).

TABLE 6·2 DECOMPOSITION OF TEL (62·5 µl l^{-1}) IN EVT MEDIUM (mg l^{-1})

* Mean of 3 experiments ±1 S.D.

Time (days)	[TEL]		[Et_3Pb^+]		[Et_2Pb^{++}]	
	Light	Dark	Light	Dark	Light	Dark
*0	0·290 ± 0·110		0·002 ± 0·002		< 0·001	
1	0·440		0·050		< 0·001	
2	0·670		0·140		< 0·001	
*3	0·967 ±0·806	1·100 ±0·930	1·590 ±1·500	0·082 ±0·041	< 0·004 ± 0·002	< 0·001 ± 0·001
5	0·430	0·230	0·770	0·090	< 0·006	< 0·001
6	0·190		0·960		< 0·015	
7	0·110	0·190	1·170	0·160	< 0·017	< 0·001

FIG 6.5 EFFECT OF 62·5 µl TEL l^{-1} ON P.MALHAMENSIS

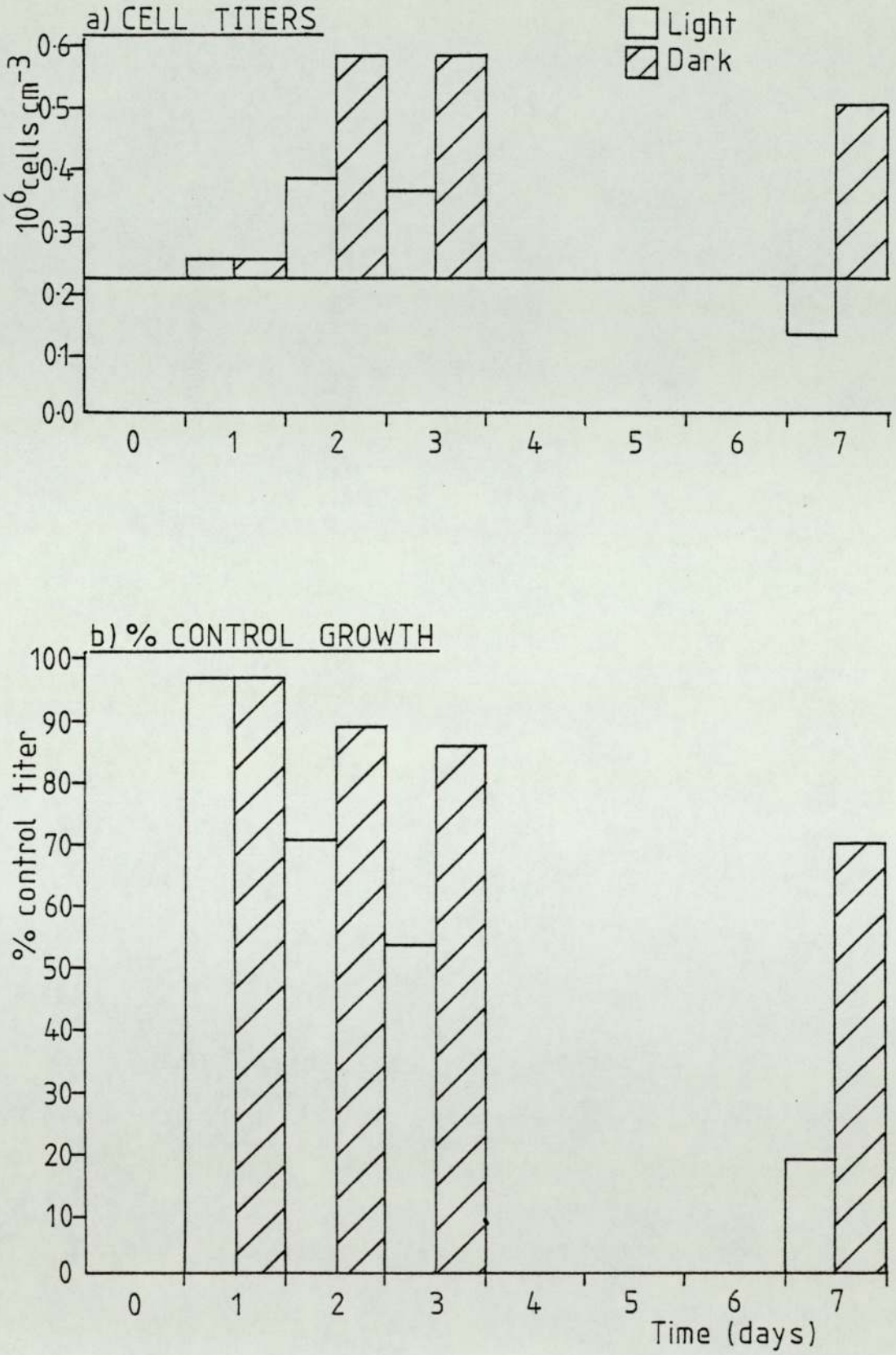
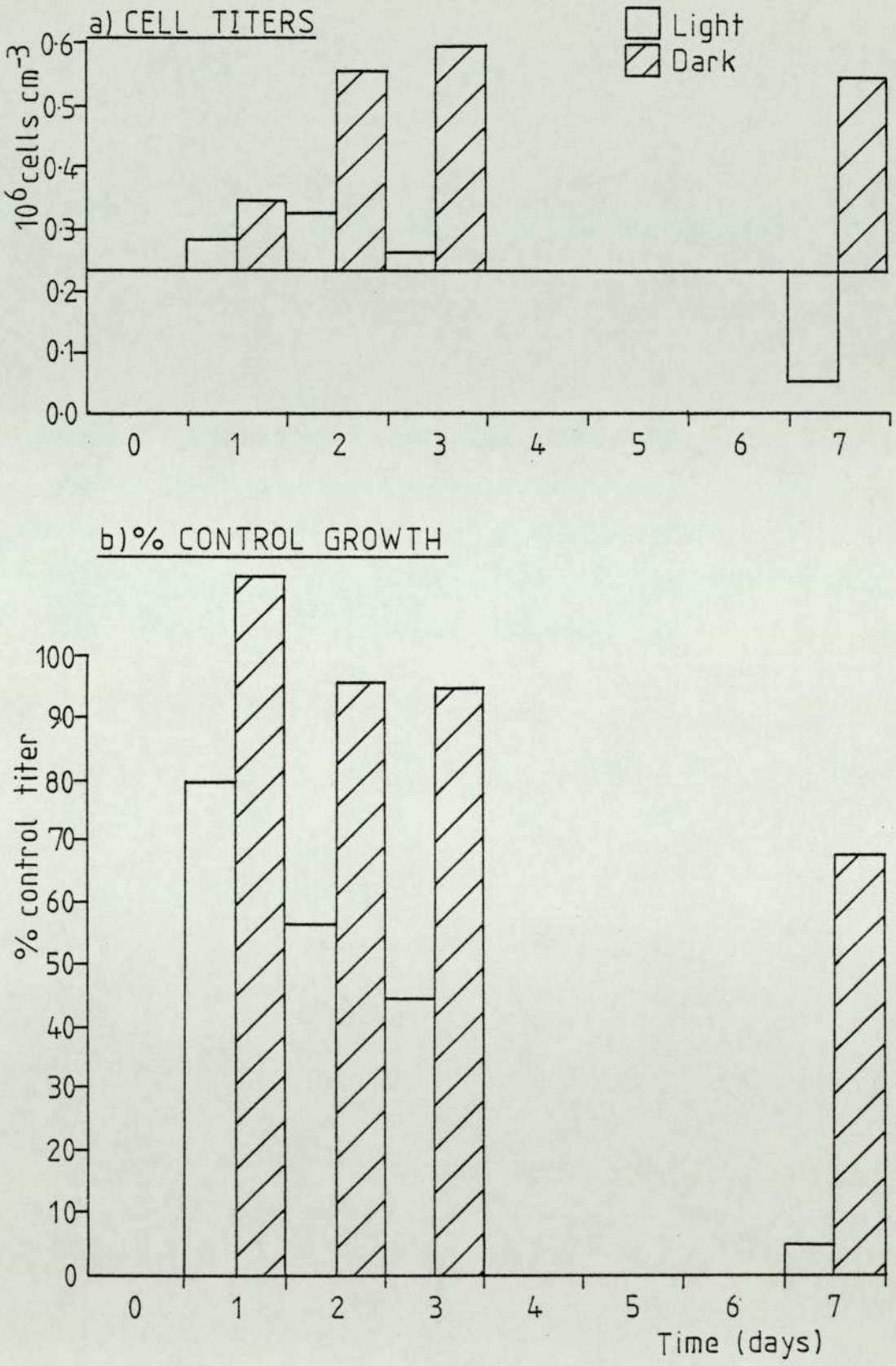


FIG 6.6 EFFECT OF 62·5 µl TEL l^{-1} ON P. MALHAMENSIS



Again both light and dark incubations suggest suspensions of TEL as concentrations are well above the solubility in aqueous media. This might have been partly due to aeration of the cultures. Microscopic droplets of TEL may have broken off from the small 5 µl pool at the bottom of the tube by the mixing action generated from the aeration.

Even with the high variability in both light and dark incubated cultures, TEL concentrations fall as time goes on. This may be a result of less and less TEL being left after the aerating action. Differences in concentration from dark and illuminated cultures are not dissimilar suggesting that decomposition in illuminated incubations, reducing TEL concentrations, was hidden by a more significant mixing process, i.e. aeration. However, Et_3Pb^+ concentrations are significantly different after 3 days with the light exposed medium containing, as expected, higher amounts. This is obviously a result of TEL photolysis. Et_3Pb^+ concentrations continue to increase with time in light incubations to extremely toxic levels, whereas in dark cultures the concentration remains low. Dark cultures show a constantly low Et_2Pb^{++} concentration suggesting that any disproportionation of TEL leading to this ion is negligible in comparison to photolytic decomposition. Slightly higher values in illuminated cultures, increasing with time, may be due to photolysis of Et_3Pb^+. All cultures show that by far the most important decomposition product is the Et_3Pb^+ ion.

After consideration of the alkyllead concentrations to which algal cells were most likely subjected, there is a strong tendency to see the falling titers as a result of Et_3Pb^+ toxicity. It is difficult to find any reason to explain the rapid decline in illuminated culture titers, whilst dark cultures fall only slowly.

The approximate concentrations of Et_3Pb^+ in both light and dark cultures

might have been expected to have caused larger titer decreases on the basis of the subsequent Et_3PbCl toxicity assays (Section 6.5). However the concentrations obtained from TEL incubations can only be rough estimates because of the variability of data. Direct comparisons between the two experiments should also be treated with caution, as the effect of Et_3Pb^+ from Et_3PbCl addition may be slightly different to that of Et_3Pb^+ in the presence of other alkyllead compounds such as TEL.

Thus all toxicity recorded from the TEL incubated cultures can be attributed to Et_3Pb^+ toxicity. This suggests very strongly that TEL in its pure form is non-toxic.

6.6.2. TML

An initial TML assay with a concentration of 62.5 µl TML l^{-1} (Fig.6.8), resulted in differences between light and dark grown cultures. Once again a more realistic picture of toxicity is gained from the percentage growth values (Fig.6.8b) rather than from actual titers (Fig.6.8a). There is a fall in dark cultures but this is less than the large drop in illuminated culture growth. The incubation was run for 2 days only, because of the limited time available for analysis at Associated Octel Company at that particular time. Also the TML from several tubes had been washed out by the aeration before 2 days. Thus no reservoir of neat TML remained in four of the ten exposed cultures.

Concentrations after 2 days incubation (Table 6.3) show a similar trend to that of TEL incubations. The main decomposition product, Me_3Pb^+, is produced at a faster rate in illuminated cultures than in dark ones but the concentrations of Me_2Pb^{++} are very low. As with TEL, accurate and consistent values for TML solubility are not available. Grove[3] reports a freshwater solubility of about 15 mg l^{-1} with lower amounts in seawater. It is interesting to note that analyses from this study indicate TML

FIG 6.7 EFFECT OF 500µl TML l^{-1} ON P.MALHAMENSIS

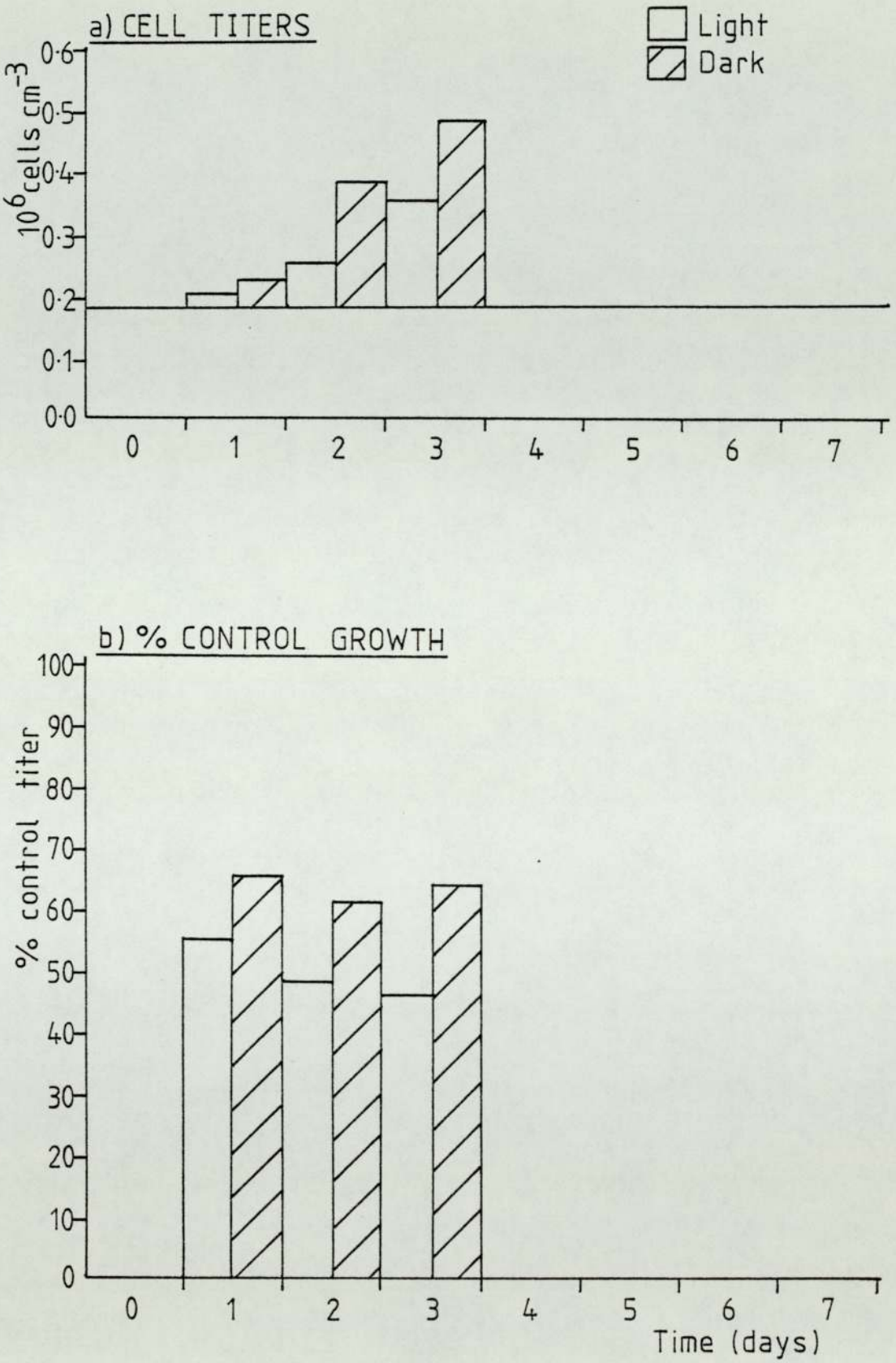
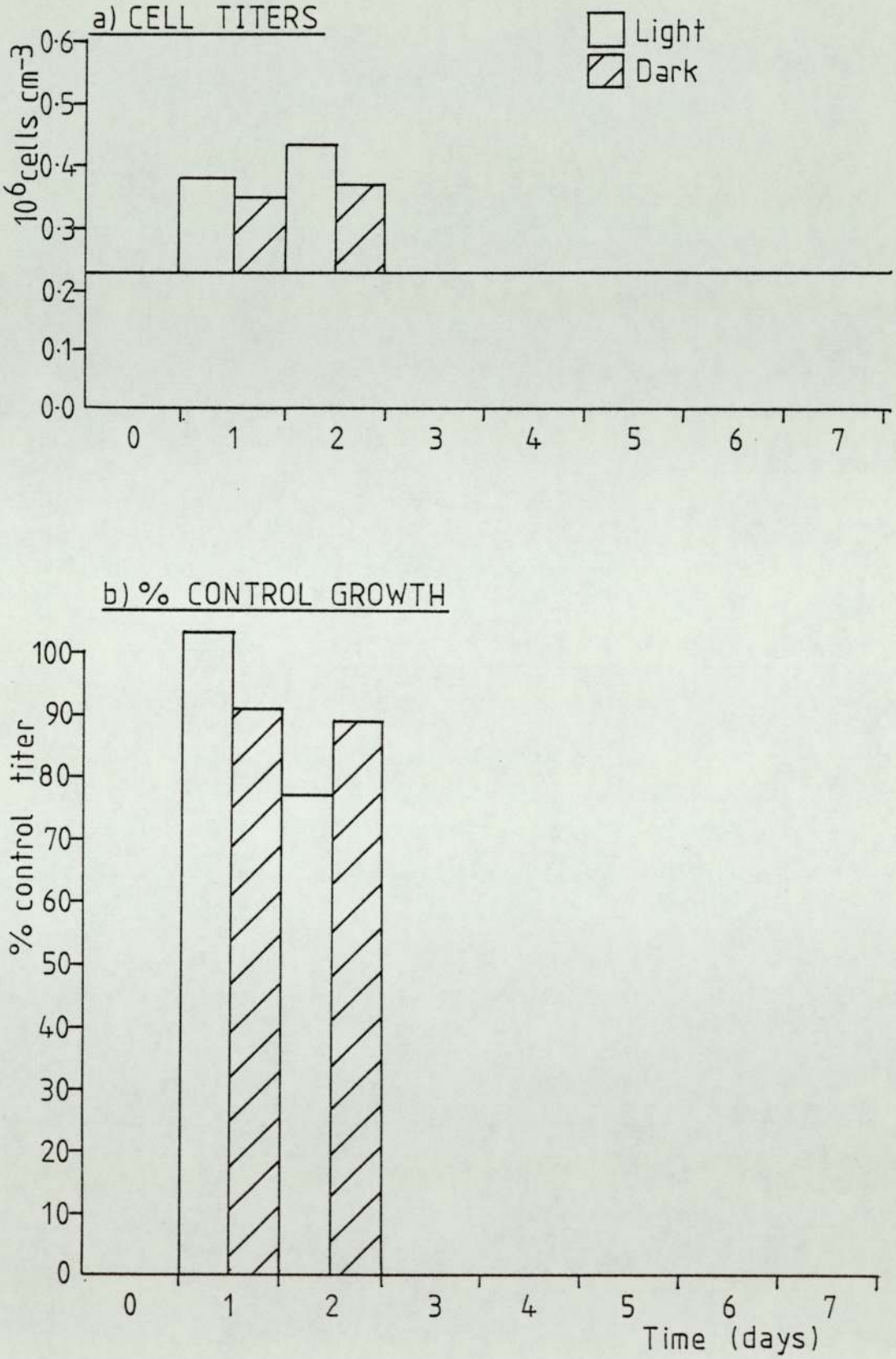


FIG 6.8 EFFECT OF 62·5µl TML l^{-1} ON P.MALHAMENSIS



concentrations to be well below the solubility given by Grove. This is in contrast to TEL where our determinations indicated greater solubility than expected from the literature values. Our results would suggest a lower solubility of TML, as insoluble reservoirs remained in the medium for long periods, presumably enabling saturation to occur. Aeration might cause high 'concentrations' due to a dispersion of the TML reservoir by mixing action. This also suggests results in this study could be above true TML solubility.

TABLE 6.3 DECOMPOSITION OF TML IN EVT MEDIUM INCUBATED IN LIGHT (L) AND DARK (D) CONDITIONS. INITIAL CONCENTRATIONS OF TML WERE 62·5 µl l^{-1} (2 DAY INCUBATION) AND 500 µl l^{-1} (3 DAY INCUBATION).

Time (days)	[TML]		[Me_3Pb^+]		[Me_2Pb^{++}]	
	L	D	L	D	L	D
2	3·64 ±2 21	2·43 ±2 93	0·29 ±0 02	0·13 ±0·02	< 0·002	< 0·002
3	< 0·02	< 0·01	* 0·83 ± 0·82	* 0·26 ± 0·05		

* combined Me_3Pb^+/Me_2Pb^{++} concentrations

A second incubation starting with 40 µl in 80cm^3 EVT medium (500 µl TML l^{-1}) was run for 3 days with similar results. TML exposed light cultures grew less well than exposed dark cultures (Fig.6.7). The higher initial TML addition was to try to maintain a reservoir of TML in the tubes without having to add more when the initial addition ran out. (Noticeably this did not occur to such an extent with TEL incubations, no doubt due to the lower volatility and solubility of TEL.) As the TML added was not completely soluble in the medium, the effects of adding a larger initial volume would not be greatly different to the first experiment. An increase in surface area of the drop might lead to higher rates of loss and possibly higher concentrations, but as these were monitored both experiments can still be compared.

The major difference between both incubations lies in the values for TML. TML after a 2 day incubation was two orders of magnitude greater than concentrations after 3 days. This was unexpected as the larger 40 µl TML reservoir should have been able to maintain at least as much TML in suspension. The analyses were carried out by two separate methods which may have resulted in some variation. The 2 day incubated samples were analysed by GLC-MS whereas the 3 day incubated samples underwent the longer hexane extraction followed by ICl/dithizone extraction (see section 2.4.1.).

Concentrations of Me_3Pb^+ after 2 days are considerably less than those of Me_3Pb^+/Me_2Pb^{++} after 3 days in both light and dark cultures. The 3 day dark value could be a result of the greater decomposition from the increased volume of TML in suspension. This is supported by the proportionally much greater concentration of Me_3Pb^+ in illuminated cultures after 3 days, than after 2 days. In other words in the light $[Me_3Pb^+]$ after 3 days is about 2.9 x$[Me_3Pb^+]$ after 2 days, but in the dark $[Me_3Pb^+]$ after 3 days is about 2.0 x$[Me_3Pb^+]$ after 2 days.

It is interesting to note that after 3 days incubation the mean [Et_3Pb^+] in illuminated cultures was about 20 x [Et_3Pb^+]in dark cultures but, after a similar period, the mean [Me_3Pb^+] in illuminated cultures was only about 2 x [Me_3Pb^+] in dark cultures. This is most likely to be due to the lower stability of TML.TEL is quite stable in the dark, but TML decomposes in both light and dark, although the light reaction is much faster[5]. This could explain the smaller difference in growth rates between illuminated and dark cultures exposed to TML rather than to TEL. However, the illuminated cultures were inhibited to a significantly greater degree than dark cultures suggesting that toxicity was due to Me_3Pb^+. As with TEL, it appears that TML in its pure form is also non-toxic.

6.6.3. Effect of Fe^{+++} on TEL Toxicity

It is known that iron (Fe^{+++}) reacts with TEL to produce the lower analogue alkyllead compounds and Fe^{++} in ethereal solution[140], and in aqueous solution in the dark[5,6]. If appreciable amounts of Et_3Pb^+ are formed by this reduction reaction, toxicity to an exposed culture of Poterioochromonas may increase. Obviously the reaction and incubation should take place in the dark to minimise photolytic decomposition of TEL and the effects of Fe^{+++} itself on the algae would have to be accounted for.

Solutions of copper (Cu^{++}) are also reduced during a reaction with TEL, resulting in Et_3Pb^+ production[5]. Markall[9] has shown Cu^{++} to be several times more reactive in bringing about TEL decomposition than Fe^{+++}. Both iron and copper are considered essential micronutrients for algae, but most are extremely sensitive to excess copper[141]. Thus, use of Cu^{++} in anything but very low concentrations would most probably be highly toxic to the algal cells. At these low concentrations significant Et_3Pb^+ production would not occur. Fe^{+++}, on the other hand, is far less

toxic to algae and even though Et_3Pb^+ production is lower than for similar Cu^{++} concentrations, significant quantities may be formed.

After four days storage of 20 mg l^{-1} Fe^{+++} + 38.7 mg l^{-1} TEL solution in the dark, Markall found 0.41 mg l^{-1} Et_3Pb^+ (as Et_2Pb^{++})(5). From our earlier investigations into Et_3Pb^+ toxicity, this concentration would result in a large reduction in growth. Values for 0 - 3 days were not reported but should still be significant. The effect of such a high iron concentration on Poterioochromonas was dramatic. Within one hour all algae had died and undergone lysis, leaving the solution clear when viewed under the microscope. Consequently a large reduction in Fe^{+++} concentration was made before further incubations were started. The effect of 1.0 mg l^{-1} Fe^{+++} can be seen in Table 6.4. The data shows Fe^{+++} to stimulate growth above control titers even though Fe^{+++} was present in EVT medium. This EVT Fe^{+++} concentration must have been limiting growth in all cultures only being alleviated by further additions.

To establish whether this stimulated growth would offset any Et_3Pb^+ toxicity, cultures were incubated with combinations of 1.0 mg l^{-1} Fe^{+++}, 62.5 µl TEL l^{-1} or neither. The resulting cell counts once more reveal the increased growth of non-TEL, Fe^{+++} exposed cultures over the double controls (Table 6.4). The effect of Fe^{+++} on the TEL incubated cultures was similar, seemingly the presence of Fe^{+++} having the major influence on growth (Fig.6.9). The cell titers of Fe^{+++}/TEL exposed tubes increased throughout the 7 day incubation, but by comparing percentage normal growth rates, it appears that rate of growth fell considerably after 2 days. The TEL-only cultures also exhibited a similar pattern. This could be due to Et_3Pb^+ production increasing toxic levels after 2 days incubation, reducing growth rate by similar amounts in Fe^{+++} dosed and non-dosed cultures. The reaction of Fe^{+++} with TEL at these concentrations

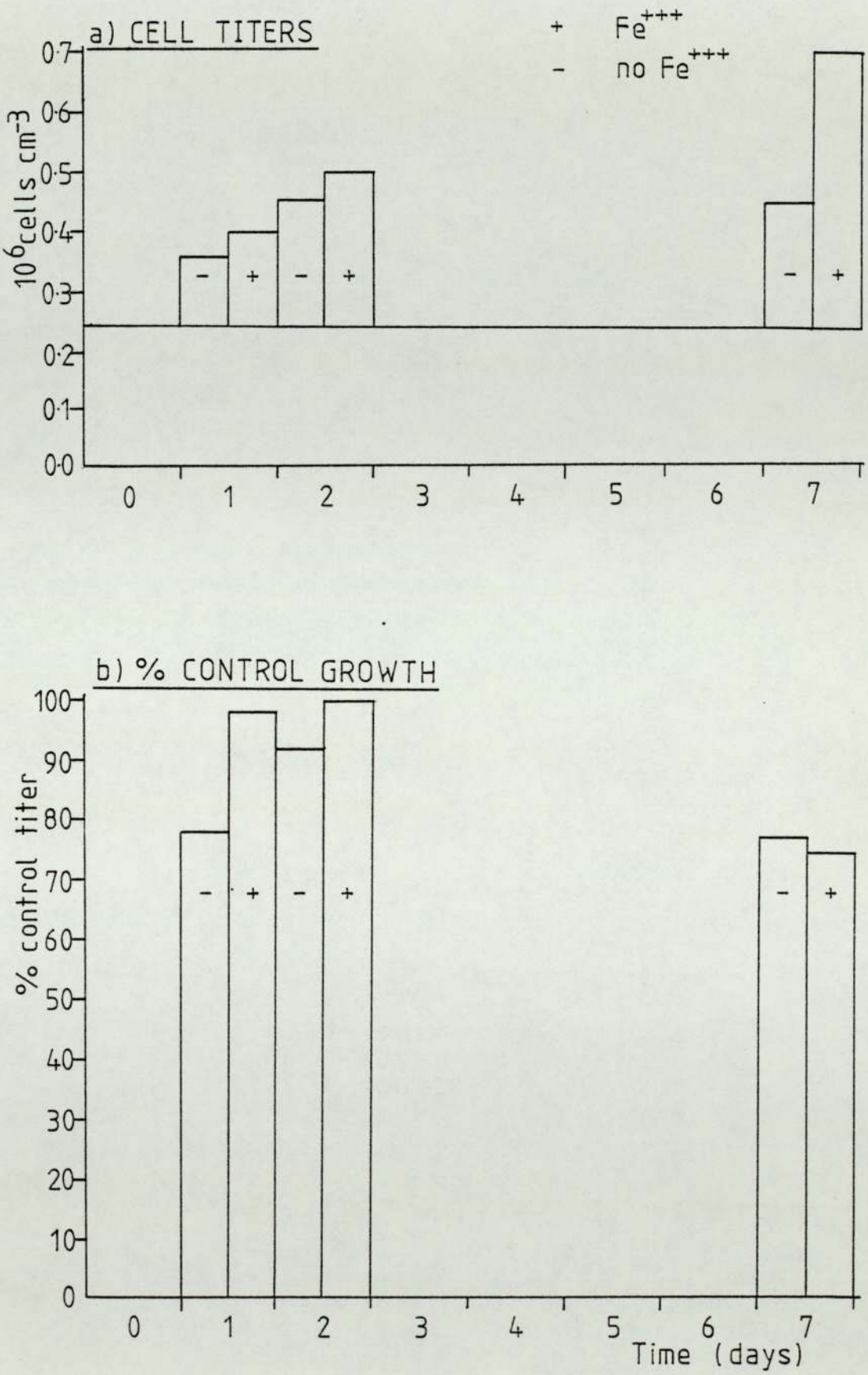
TABLE 6.4 EFFECT OF Fe^{+++} ON TITERS OF CONTROL AND TEL EXPOSED POTERIOOCHROMONAS CULTURES INCUBATED IN THE DARK

[TEL] = 62·5µl l^{-1}

[Fe^{+++}]= 1·0 mg l^{-1}

TIME (DAYS)	TITER (10^6cells cm^{-3}) (± 1 S.D.)			
	TEL	TEL + Fe^{+++}	Control	Control+Fe^{+++}
0			0·20 ± 0·05	0·20 ± 0·04
1			0·22 ± 0·08	0·31 ± 0·06
3			0·38 ± 0·08	0·45 ± 0·07
5			0·40 ± 0·10	0·53 ± 0·10
0	0·23 ± 0·05	0·23 ± 0·05	0·23 ± 0·05	0·23 ± 0·05
1	0·35 ± 0·07	0·39 ± 0·04	0·45 ± 0·03	0·40 ± 0·03
2	0·44 ± 0·10	0·49 ± 0·03	0·48 ± 0·02	0·49 ± 0·02
7	0·44 ± 0·08	0·69 ± 0·09	0·57 ± 0·08	0·93 ± 0·13

FIG 6.9 EFFECT OF 62·5µl TEL l^{-1} ± 1·0mg l^{-1} Fe^{+++} ON P. MALHAMENSIS



therefore appears not to materially influence toxicity but generally stimulates growth.

6.7. Accumulation of Alkyllead Compounds by Poterioochromonas

Analyses of the total algal content of exposed cultures revealed some surprising accumulation of the various alkyllead species in EVT medium. These results were mainly associated with TAL exposed cultures. But first the accumulation of trialkyllead compounds will be considered.

6.7.1. Accumulation from Et_3PbCl and Me_3PbCl Exposure

After 3 days incubation in light and dark exposed to 0.1 mg l^{-1} Et_3PbCl or Me_3PbCl, all of the algal cells were extracted and analysed as outlined in Section 6.3.1. The EVT medium was also analysed for soluble alkyllead. The results of these two incubations are given in Tables 6.5 and 6.6.

No TAL from either set of incubations was detected above the limits of 0.005 mg l^{-1} in EVT medium or algae. If any TAL had been present, it would have been produced by trialkyllead disproportionation or chemical alkylation, or by alkylation caused by the algae. As all of these possibilities would result in very low yields and because of the insoluble and volatile nature of TAL, it is not surprising that no TAL was detected. Thus in Et_3PbCl exposed cultures only Et_3Pb^+ and Et_2Pb^{++} were found in the cells and in solution, and only Me_3Pb^+ and Me_2Pb^{++} were found in Me_3PbCl exposed cultures. Results for algal accumulation are expressed as $\mu g/10^6$ cells to show actual amounts of lead absorbed, and as a percentage of the total amount of the particular species present at the time of analysis in the EVT medium.

The dialkyllead results for medium and algae are less accurate than the trialkyllead values because of the lower analogue levels of the former.

TABLE 6.5 CONCENTRATIONS OF ALKYLLEAD IN ALGAE AND MEDIUM AFTER 3 DAYS EXPOSURE TO 0·1 mg l^{-1} Me_3PbCl

(L) – Light incubation (D) – Dark incubation

(c) – Control

Culture	±1S.D. Final Titer 10^6 cell/cm^3	EVT MEDIUM			ALGAE			
		Me_3Pb^+ mg l^{-1}	Me_2Pb^{++} mg l^{-1}	(1) Total Me_3Pb^+ µg	(2) Total Me_2Pb^{++} µg	(3) (×1000) Me_3Pb^+ µg/10^6cell	(4) (×1000) Me_2Pb^{++} µg/10^6cell	$\frac{(3)}{(1)} \times \frac{100}{1000}$	$\frac{(4)}{(2)} \times \frac{100}{1000}$
1 (c.L)	0·71 ± 0·11	< 0·001	< 0·001			< 0·1	< 0·1		
2 (L)	0·45 ± 0·06	0·094	< 0·002	7·55	< 0·12	1·3	< 0·2	0·017	0·165
3 (L)	0·56 ± 0·08	0·098	< 0·002	7·88	< 0·11	1·6	< 0·2	0·020	0·174
4 (L)	0·49 ± 0·04	0·110	< 0·003	8·81	< 0·25	1·8	< 0·2	0·020	0·079
5 (D)	0·57 ± 0·09	0·106	< 0·002	8·46	< 0·12	1·2	< 0·1	0·014	0·084
6 (D)	0·49 ± 0·02	0·125	< 0·002	9·99	< 0·13	1·3	< 0·2	0·013	0·159
7 (c.D)	0·54 ± 0·09	< 0·002	< 0·001			< 0·1	< 0·1		

TABLE 6.6 CONCENTRATIONS OF ALKYLLEAD IN ALGAE AND MEDIUM AFTER 3 DAYS EXPOSURE TO 0·1mgl^{-1} Et_3PbCl

(L) – Light incubation (D) – Dark incubation

(c) – Control * – not included in calculations

Culture	±1 S.D. Final Titer 10^6 cell/cm^3	EVT MEDIUM (1) Et_3Pb^+ mg l^{-1}	EVT MEDIUM Et_2Pb^{++} mg l^{-1}	EVT MEDIUM (1) Total Et_3Pb^+ µg	EVT MEDIUM (2) Total Et_2Pb^{++} µg	ALGAE (3) (×1000) Et_3Pb^+ µg/10^6cell	ALGAE (4) (×1000) Et_2Pb^{++} µg/10^6cell	$\frac{(3)}{(1)} \times \frac{100}{1000}$	$\frac{(4)}{(2)} \times \frac{100}{1000}$
1 (c.L)	0·77 ± 0·10	< 0·001	< 0·001			< 0·001	< 0·01		
2 (L)	0·80 ± 0·07	0·086	< 0·002	6·88	< 0·13	1·6	< 0·6	0·02	0·45
3 (L)	0·79 ± 0·11	0·074	< 0·002	5·95	< 0·12	1·5	< 0·5	0·03	0·46
4 (L)	0·77 ± 0·15	0·076	< 0·001	6·08	< 0·03	2·1	< 0·1	0·03	0·52
5 (L)	0·70 ± 0·12	0·065	< 0·002	5·18	< 0·14	1·9	< 1·2	0·04	0·87
6 (L)	0·78 ± 0·05	0·068	< 0·002	5·46	< 0·12	2·0	< 0·1	0·04	0·08
7 (c.D)	0·74 ± 0·10	< 0·001	< 0·001			< 0·001	< 0·001		
8 (D)	0·91 ± 0·09	0·077	* 12·1	6·12		1·0	< 2·1	0·02	0·21
9 (D)	0·91 ± 0·09	0·104	< 0·002	8·35	< 0·13	2·2	< 2·3	0·03	1·71
10 (D)	0·98 ± 0·06	0·098	< 0·002	7·87	< 0·13	1·9	< 2·2	0·03	1·64
11 (D)	1·08 ± 0·14	0·110	< 0·002	8·82	< 0·14	1·3	< 1·8	0·01	1·32
12 (D)	0·83 ± 0·13	0·091	< 0·002	7·31	< 0·12	1·1	< 2·0	0·02	1·74

Aqueous concentrations of both dialkyls were calculated as 'less than' values by the polarograph computer. In other words, the peaks obtained from ASV were so low in comparison to the standard peak (see Section 2.6) that the concentrations could only be quoted to an accuracy of less than a maximum value. Although not necessarily related to aqueous concentrations algal dialkyllead contents were also too low to be given precise figures. Thus, accumulated dialkyllead calculated as a percentage of the total dialkyllead in solution must be regarded as very rough estimates, and more importance should be placed upon orders of magnitude rather than actual figures.

Et_3Pb^+ accumulation is very similar to Me_3Pb^+ accumulation, comparing values of $\mu g/10^6$ cells (Tables 6.6 and 6.5). With the limited amount of data available it is difficult to compare light and dark cultures, but from what data there are it appears that illumination does not greatly influence uptake. Each million cells contains approximately 0.01% - 0.04% of the total mass of trialkyllead in solution, for both Et_3Pb^+ and Me_3Pb^+. Et_3Pb^+ is possibly accumulated proportionally slightly more than Me_3Pb^+.

The corresponding dialkyllead values are more varied. Insufficient data are available for detection of trends within light and dark cultures exposed to Me_3Pb^+ (Table 6.5), but higher percentage accumulation of Et_2Pb^{++} is present in dark incubated cultures than in illuminated ones (Table 6.6). This is also indicated by the higher Et_2Pb^{++} content of dark cells than illuminated cells. Concentrations of Et_2Pb^{++} in EVT medium do not show a similar difference between light and dark conditions even though this may have been the case if photolysis of Et_3Pb^+ had occurred significantly.

It is notable that percentage accumulation of both dialkyllead compounds is generally an order of magnitude greater than corresponding percentage accumulation of trialkyllead. This could be due to preferential dialkyllead

uptake over trialkyllead, or a conversion of trialkyllead to dialkyllead within the cell. Consideration of the higher amounts of Et_2Pb^{++} in dark incubated cells (Table 6.5) tends to support the possibility of some sort of metabolism of alkyllead by the cell. This would not be unexpected, for it is well documented in the literature that many organisms convert toxic compounds into forms less deleterious to the cell. The different metabolic pathways in operation within a cell when photosynthesising, or growing in a dark regime could influence alkyllead metabolism, giving different rates of conversion under the two conditions.

If metabolism of Et_3Pb^{+} to Et_2Pb^{++} does occur, a further dealkylating step, Et_2Pb^{++} to Pb^{++}, might also be present. Because of the nature of the analytical procedure, it was not possible to determine inorganic lead levels within the algae. Therefore an increase in Pb^{++} resulting from complete dealkylation cannot be shown.

6.7.2. Accumulation from TEL Exposure

Accumulation of TEL by algal cells could only be determined approximately because of the previously discussed inaccuracies of the extraction/analytical procedure. For this reason, and because the total amount of TEL in the medium (in solution/suspension and in the reservoir) was unknown, calculations of percentage algal accumulation have not been made.

Two separate incubations of TEL exposed cultures were analysed. The first incubation involved algae exposed to 50 µl TEL/l and analyses were conducted after 5 to 7 days incubation (Table 6.7). The second assay contained 62.5 µl TEL/l, analyses being conducted after 3 days exposure (Table 6.8). Apart from the difference in TEL volume, all other conditions were similar, both incubations being assessed at the Associated Octel Company. The difference in initial TEL doses is not particularly important as the droplets would be of similar size, both forming insoluble

TABLE 6.7 CONCENTRATIONS OF ALKYLLEAD IN ALGAE AND MEDIUM EXPOSED TO 50µl l^{-1} TEL

Symbols as for table 6.8

Culture	Final Titer 10^6 cell/cm^3	Length of incubation days	EVT MEDIUM TEL mg l^{-1}	Et_3Pb^+ mg l^{-1}	Et_2Pb^{++} mg l^{-1}	(1) Total Et_3Pb^+ ug	(2) Total Et_2Pb^{++} ug	ALGAE (3) TEL ug/10^6cell	(3) Et_3Pb^+ ug/10^6cell	(4) Et_2Pb^{++} ug/10^6cell	$\frac{(3)}{(1)} \times \frac{100}{1}$	$\frac{(4)}{(2)} \times \frac{100}{1}$
1 (c.L)	1·07	7	< 0·01	< 0·001	< 0·001			< 0·01	< 0·001	< 0·001		
2 (L)	0·93	5	0·43	0·77	< 0·007	46·4	< 0·38	0·01	0·129	0·039	0·28	10·3
3 (L)	0·79	6	0·19	0·96	< 0·015	57·7	< 0·93	2·51	0·314	0·092	0·20	9·90
4 (L)	0·75	7	0·11	1·17	< 0·017	70·4	< 1·01	*10·74	*1·336	*0·159	1·89	15·7
5 (cD)	0·74	8	< 0·01	< 0·001	< 0·001			< 0·01	< 0·001	< 0·001		
6 (D)	0·62	5	0·23	0·09	< 0·001	5·69	< 0·02	1·23	0·049	0·017	0·86	85·0
7 (D)	0·82	7	0·19	0·16	< 0·001	9·36	< 0·04	0·95	0·147	0·083	1·57	208

TABLE 6.8 CONCENTRATIONS OF ALKYLLEAD IN ALGAE AND MEDIUM AFTER 3 DAYS EXPOSURE TO 62·5µl TEL l^{-1}

(L) - Light incubation (D) - Dark incubation

(c) - Control * Probable TEL contamination

Culture	± 1 S.D. Final Titer 10^6 cells/cm^3	EVT MEDIUM TEL mg l^{-1}	Et_3Pb^+ mg l^{-1}	Et_2Pb^{++} mg l^{-1}	(1) Total Et_3Pb^+ µg	(2) Total Et_2Pb^{++} µg	ALGAE (3) TEL µg/10^6cells	(3) Et_3Pb^+ µg/10^6cells	(4) Et_2Pb^{++} µg/10^6cells	(3)/(1) x 100	(4)/(2) x 100
1 (L)	0·24 ± 0·05	1·800	3·229	< 0·004	258	< 0·28	0·526	1·15	0·09	0·45	32·3
2 (L)	0·31 ± 0·04	0·190	0·282	< 0·007	22·6	< 0·55		2·95	0·18	13·06	33·4
3 (L)	0·30 ± 0·05	0·910	1·259	< 0·003	100	< 0·24	3·77	0·95	0·06	0·95	26·2
5 (D)	0·41 ± 0·06	0·280	0·033	< 0·002	2·64	< 0·13	0·008	0·17	0·03	6·33	23·0
6 (D)	0·36 ± 0·07	1·700	0·140	< 0·002	11·2	< 0·14	*21·96	*2·46	*0·14	21·99	94·4
7 (D)	0·28 ± 0·05	2·000	0·064	0·001	5·114	< 0·04	0·050	0·17	0·06	3·31	150
8 (D)	0·42 ± 0·04	2·100	0·047	< 0·002	3·79	< 0·02	2·56	0·49	0·08	12·96	64·7
9 (cD)	0·53 ± 0·09	< 0·01	< 0·001	0·001			< 0·005	< 0·005	< 0·001		

pools at the bottom of culture tubes. A more significant factor is the different times of incubation. The longer the incubation, the longer the TEL decomposition, especially in illuminated cultures, and consequently the greater the concentration of Et_3Pb^+.

The TEL concentrations recorded in EVT medium are highly variable, with several values indicating a suspension rather than a true solution (Table 6.7 and 6.8). There is no obvious difference between the amounts of TEL in dark and illuminated cultures as might be expected from the constant source of liquid TEL at the bottom of each exposed tube.

Generally the amounts of TEL found in the medium from the second incubation are greater than those from the first. This could be due to the larger initial TEL addition, but is more likely to have been influenced by the longer incubations of the first assay, possibly enabling some equilibration to the true TEL solubility. On the other hand slight differences in the positioning of the aerating pipette tips could lead to large differences in the amount of TEL taken into suspension or solution.

TEL values associated with the biological material are even more highly varied. Culture 6 (Table 6.8), with an accumulation of 20 $\mu g/10^6$ cells, and Culture 4 (Table 6.7) with 10 $\mu g/10^6$ cells, were most probably the result of small droplets of neat TEL taken through the extraction procedure causing erroneous values. Insufficient data are available for a detailed discussion, and from the figures obtained it is difficult to establish the uptake of TEL by cells because of large analytical errors. However TEL absorption can be inferred from the high Et_3Pb^+ and Et_2Pb^{++} accumulation.

After 3 days incubation, concentrations of Et_3Pb^+ and most probably Et_2Pb^{++} are higher in illuminated cultures (Table 6.8). Similar higher

values in illuminated cultures are also found after 5 and 7 days (Table 6.7). This is to be expected for the obvious reasons of TEL photolysis. The algal accumulation of both degradation products also follows the trend in both incubations. Once again the atypical values for Culture 6 (Table 6.8) are probably due to TEL contamination of the cells. Although only single values are available for longer incubated accumulation (Table 6.7) by Poterioochromonas, this data suggests that accumulation increases with time. However, comparing the first incubation (Table 6.7) with the second reveals that the shorter incubation yielded algae with the higher accumulation. Similar comparisons for medium concentrations of Et_3Pb^+ and Et_2Pb^{++} do not follow this pattern as generally the first and longer incubations contain greater concentrations than the second 3 day incubations. The unexpected algal accumulation might, therefore, be due to some external factor such as differences in extraction efficiency between the two periods.

It is interesting to consider algal accumulation when expressed as a percentage of the total amount of Et_3Pb^+ or Et_2Pb^{++} present in the medium, i.e:

$$\frac{Et_3Pb^+ \text{ algae } (\mu g/10^6 \text{ cells})}{Et_3Pb^+ \text{ in medium } (\text{ total amount } \mu g)} \times \frac{100}{1}$$

Percentage accumulation values for Et_3Pb^+ and Et_2Pb^{++} from both incubations are generally higher in dark grown cultures than in illuminated ones. This suggests that accumulation of these compounds is not proportional to the respective concentrations in aqueous solution at the time of analysis. Of course, the final titers affect the amount of alkyllead per million cells, but because these titers are not widely spread this influence is of minor importance. Actual accumulation is predominantly greater in light grown cells exposed to higher concentrations of Et_3Pb^{++} and Et_2Pb^{++}, but dark grown cells contained proportionally more alkyllead when compared

to the total amounts of the compound in 80cm^3 of the medium.

The most likely explanation is that after absorption of TEL, the cells dealkylate the compound in some metabolic process. This leads to Et_3Pb^+ and Et_2Pb^{++} formation. The dealkylation sequence:

$$TEL \rightarrow Et_3Pb^+ \rightarrow Et_2Pb^{++} \rightarrow Pb^{++}$$

which is known to occur by chemical processes[12], cannot be substantiated from the data, but remains a likely possibility. Uptake of Et_3Pb^+ from solution would be metabolised by the same sequence. If dealkylation was not a rapid process, with Et_3Pb^+ and Et_2Pb^{++} remaining stable for some time, then it would not be possible for algal accumulation to reach a high level in proportion to the amounts of alkyllead species in solution. Thus, in dark grown cultures where no photolytic decomposition occurred, minimising Et_3Pb^+ and Et_2Pb^{++} concentrations, proportionally more alkyllead would be contained by the cells than in the light grown cultures. This seems to be the only logical explanation of the dark grown cells Et_2Pb^{++} content exceeding the total amount in solution (Tables 6.7 and 6.8). TEL metabolism within the cell is also implicated by comparisons of Et_3Pb^+ uptake by dark grown, TEL exposed cells. e.g. Table 6.8, with uptake by dark grown, Et_3Pb^+ exposed cells, Table 6.6. Although similar Et_3Pb^+ concentrations were present in both incubations (0.01 mg l^{-1} to 0.1 mg l^{-1}), cells from TEL incubations contained amounts of Et_3Pb^+ two orders of magnitude greater than Et_3Pb^+ exposed incubations. This greater Et_3Pb^+ uptake most likely occurred as a result of TEL metabolism within the cell.

6.7.3. Accumulation from TML Exposure

For reasons previously stated, analyses of TML and decomposition products were conducted after 2 days incubation. Analyses of Me_3Pb^+ and Me_2Pb^{++} contents of Poterioochromonas are available for light and dark grown cultures. However, TML algal data are not available. This loss is not

particularly disturbing because similar inaccuracies in TEL algal analyses apply to TML analyses.

Aqueous concentrations of Me_3Pb^+ (Table 6.9) are more consistent than corresponding Et_3Pb^+ values from TEL incubations (Table 6.8), as are the TML determinations. Again the TML content of EVT medium does not vary greatly between light and dark cultures, but there is an unexpected greater concentration of Me_3Pb^+ in illuminated cultures. Unlike Et_2Pb^{++} in TEL assays, a similar trend is not evident for Me_2Pb^{++}.

Accumulation of Me_3Pb^+ is more variable than corresponding concentrations in solution. The two atypical values from Culture 4 and Culture 10 (Table 6.9) are once more likely to be the result of TML contamination during extraction/analytical procedures. There is no obvious difference in accumulation between illuminated and dark cultures as found in the Et_3Pb^+ accumulation. This is in spite of higher exposure of illuminated cultures to Me_3Pb^+. Me_2Pb^{++} accumulation also does not support such a difference.

Comparing accumulation of Me_3Pb^+ in dark conditions from TML exposed incubations (Table 6.9) with that from Me_3Pb^+ exposed incubations (Table 6.5) shows that greater Me_3Pb^+ contents are found in the TML exposed cells. As both assays involved similar aqueous concentrations of Me_3Pb^+ after two days, TML uptake is implied. This situation is similar to that found in TEL and Et_3Pb^+ exposed incubations.

The algal accummulation of Me_3Pb^+ and Me_2Pb^{++} as a percentage of the total amount of the respective compounds in solution does not exhibit the obvious trend seen in TEL incubations, of greater proportional accumulation by dark grown cells. Again the limited data do not give a clear picture of the relationships. It is not possible, therefore, to

TABLE 6.9 CONCENTRATIONS OF ALKYLLEAD IN ALGAE AND MEDIUM AFTER 2 DAYS EXPOSURE TO 62·5µl TML l^{-1}

(L) – Light incubation (D) – Dark incubation

(c) – Control * – Probable TML contamination

Culture	Final Titer 10^6 cells/ml	EVT MEDIUM TML mg l^{-1}	Me_3Pb^+ mg l^{-1}	Me_2Pb^{++} mg l^{-1}	(1) Total Me_3Pb^+ µg	(2) Total Me_2Pb^{++} µg	ALGAE TML ug/10^6 cell	(3) (×1000)+ Me_3Pb^+ ug/10^6 cell	(4) (×1000) Me_2Pb^{++} ug/10^6 cell	(3) 100/(1) ×1000	(4) 100/(2) ×1000
1(c.L)	0·37 ±	0·10	<0·001	<0·001				<0·001	<0·001		
2(L)	0·45 ±	3·00	0·296	0·010	23·7	<0·79		30	<0·18	0·13	<0·02
4(L)	0·43 ±	7·42	0·298	<0·002	23·8	<0·13		1128*	16·3*	4·7	12·5
5(L)	0·39 ±	2·62	0·281	<0·002	22·4	<0·13		7·8	<0·19	0·04	0·15
6(L)	0·38 ±	2·54	0·267	<0·002	21·3	<0·13		26·0	1·67	0·12	1·28
7(cD)	0·37 ±	<0·01	<0·001	<0·001				<0·001	<0·001		
8(D)	0·38 ±	2·21	0·101	<0·002	8·1	<0·12		19·0	<0·19	0·24	0·17
9(D)	0·36 ±	2·41	0·144	<0·001	11·6	<0·12		13·0	1·13	0·12	1·00
10(D)	0·37 ±	2·85	0·140	<0·002	11·2	<0·12		189*	2·27*	1·69	1·89
11(D)	0·33 ±	2·25	0·123	<0·002	9·8	<0·12		38·3	<0·30	0·39	0·25

consider TML metabolism from this aspect of the data, although it is probable from the earlier discussed data comparisons.

Because of the difference in incubation periods between TEL and TML exposed cultures, comparative uptake of respective methyl and ethyl compounds is not easily discussed. Even though exposure concentrations of Et_3Pb^+ and TEL (Table 6.8) were lower than Me_3Pb^+ and TML (Table 6.9), Poterioochromonas appears to accumulate ethyllead compounds to a greater extent. This could be explained by the extra 24 hours of ethyl lead accumulation, TML exposed cultures only receiving 2 days incubation. However, the accumulation of Et_3Pb^+ was at least an order of magnitude greater than accumulation of Me_3Pb^+. Whether an extra day of incubation could account for this large difference is questionable. Certainly from the varying incubation periods of TEL exposed cultures (Table 6.7), such an increase between 5 days and 6 days incubation was not apparent. Support for a preferential uptake or accumulation of ethyl lead compounds was also suggested by the trialkyllead exposed cultures (Tables 6.5 and 6.6).

An enhanced accumulation of ethyl lead compounds as opposed to methyllead compounds would not be unexpected because of the longer alkyl chain length being more lipid soluble or lipophilic, thereby making uptake easier. It would be interesting to determine the relative accumulation of a range of alkylleads of varying chain length to see if this parameter does exhibit a strong influence.

CHAPTER SEVEN

DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

7.1. Introduction

The probable lethal poisoning of large numbers of coastal birds by alkyllead compounds in 1977[52], highlighted the lack of knowledge we have about the fate and toxicity of such compounds in natural systems. An understanding of the biology of estuarine organisms, combined with the chemical properties and behaviour of alkyllead species, provides the basis for an insight into how such an incident could have arisen. By understanding laboratory investigations into the relationships between these compounds and the biota, some of this understanding may be achieved.

7.2. Toxicity to Bivalves

From our investigations into the toxicity of some common environmentally occurring alkyllead species to bivalves, some general inferences can be made. Static tests are of limited value even when frequent medium changes are made and analytical data available. TAL compounds especially are not satisfactorily tested by such procedures. For these highly volatile and insoluble materials, continuous flow systems such as those used by Maddock and Taylor[51], are more appropriate in maintaining reasonably constant exposure concentrations. Less volatile and relatively soluble trialkyllead compounds are more suitably tested under static conditions, as their concentrations are less affected by aeration or illumination.

Acute toxicity tests (static or continuous flow), such as the static assays undertaken in Chapter 3, only yield data related to the lethality of alkyllead compounds. Longer term chronic tests determining sub-lethal effects on parameters of behaviour or fecundity, for example, would be important aspects in the overall assessment of environmental alkyllead toxicity. These tests were not investigated in the present study because

of the lack of necessary continuous flow facilities.

Comparisons of our data with the only other study which has investigated toxicity to bivalves[51], suggests that Mytilus edulis is more sensitive than Scrobicularia or Macoma to all of the compounds tested common to both studies. 96 hour LC_{50} values determined after Mytilus exposure are: 1.1 mg l^{-1} Et_3PbCl, 0.5 mg l^{-1} Me_3PbCl, 0.1 mg l^{-1} TEL. None of these concentrations caused 50% mortality of Macoma or Scrobicularia within 96 hours. However, this may be due to the previously discussed avoidance behaviour, rather than toxicological activity. As no mention of valve adduction was made by Maddock and Taylor, and because 96 hour LC_{50} values were obtained (i.e. short term mortalities), Mytilus was presumably continuously exposed to the toxicant from the beginning of the assay. On the contrary Scrobicularia and Macoma did exhibit valve closure, and were most probably abstaining from alkyllead exposure for some periods. Thus even if Mytilus was of similar sensitivity, it would be expected to appear less tolerant if times to 50% mortality are considered.

One of the advantages of undertaking tissue preparation assays, as with the siphonal exposures, is that such behavioural complications are avoided. Although the toxicity values are not likely to be highly predictive of true environmental toxicities to whole animals, the preparations are extremely useful in determining comparative toxicity. Siphonal preparations are far less variable than whole animals and have a narrower range of responses which enables easy comparisons of various alkyllead compounds.

As 96 hour LC_{50} values were not obtained for Macoma or Scrobicularia, comparisons of toxicity with Mytilus can only be general. However, relative toxicities of the various alkyllead compounds can be compared. Although Maddock and Taylor generally found ethyl species to be more

toxic than the corresponding methyl lead compounds, Mytilus was an exception to this trend being slightly more tolerant to Et_3PbCl than Me_3PbCl (96 hour LC_{50} values are: 0.5 mg l^{-1} Me_3PbCl, 1.1 mg l^{-1} Et_3PbCl). Data from Scrobicularia and Macoma exposures to trialkylleads suggest that the longer chain length groups are more toxic. Indeed this finding emerged from every comparative assay (including algal) undertaken in this study.

Bioaccumulation in bivalves in the present study was not investigated extensively. Again, because of the lack of suitable facilities and also because of the considerable and continuous analytical effort required, long-term sub-acute exposures were impractical. Analyses of animals involved in acute tests provided a useful indication as to the distribution of alkyllead compounds within individual organs or tissue areas. Data for actual whole body accumulation are not detailed and should only be interpreted as indicating that alkyllead accumulates by some factor above exposure concentrations.

Accumulation in Mytilus edulis[51] was more properly studied, and alkyllead distribution within individuals was also determined. Mytilus, being a suspension feeder, accumulated the compounds in organs in the following abundances:

gill > digestive gland > foot > gonad

The greatest loading occurs in the gill emphasising the importance this organ plays in filtration. The corresponding relative organ loading for Scrobicularia is:

digestive gland > gill = rest = siphon

(Macoma were too small to yield sufficient tissue for analysis.) Thus, the digestive gland in Mytilus accumulates more alkyllead than both foot and gonad, (these latter organs corresponding to rest in Scrobicularia), this also being true for Scrobicularia. However, in Scrobicularia the

the digestive gland contained the greatest loading rather than the gill. This is possibly due to algal accumulation in the static system reaching and being analysed in the digestive gland. Although the period of Phaeodactylum exposure is unknown, it would probably not exceed several hours because of the filtering activity of the molluscs. However even in this short period significant concentrations of alkyllead may have accumulated to a greater extent than in the phytoplankton in the flow-through system of Mytilus assays where natural seawater was used. There are also the fundamental feeding differences between the two bivalves. Even though both animals obtained food from suspension feeding, Scrobicularia is preferentially a deposit feeder, possibly resulting in different alkyllead loadings to the suspension feeding Mytilus. The lack of sediment may have influenced the uptake rate of alkyllead in some unkown way, producing accumulation or toxicity responses different to that of an animal ingesting substantial amounts of solid material.

The estimations of alkyllead toxicity bear little relation to effects in the natural environment. The enormous biological and chemical complexity of what might be considered as a relatively simple, homogeneous environment cannot be realistically controlled or analysed. Too many variable parameters operate. By using as near as possible to totally controlled conditions in the laboratory, effects of alkyllead compounds may be determined, but these should not be used to predict directly the toxicity in the natural system. Such studies provide only an approximate guess as to what occurs to bivalves in estuarine substrates, even when as many parameters effects as possible are included in such predictions. For example, the rapid adsorption of alkyllead into sediments has been demonstrated in this study (Section 3.3.) and in others[5,25]. This would have a significant influence on a bivalve population inhabiting such sediments. Although a slow decomposition of adsorbed alkyllead

would be expected[5], this sediment so contaminated might release or desorb the toxic material. Biologically available and active alkyllead species might then be concentrated through bioaccumulation, causing deleterious effects in the molluscs. In other words, sediments could, in certain circumstances, concentrate alkyllead from overlying waters, thereby increasing the exposure levels of bivalves consuming the sediment.

The abundance of sulphide and various iron species in estuarine sediment could favour the disproportionation of trialkyllead into TAL and inorganic lead[25]. Our results support the theory of sulphide reduction of trialkyllead to TAL and inorganic lead, albeit indirectly through toxicity data. In sediments where sulphide is in excess, and there is a significant amount of catalysing species such as Fe^{++},(i.e. in the majority of estuarine sediments) the overall reaction for all alkyllead compounds would be the formation of inorganic lead.

Thus the greater the number of parameters whose reactions or behaviour with alkyllead are understood simplistically through laboratory experiments, the closer to the real situation we get. Of course interactions between these parameters and how they affect the fate of alkyllead are the essential next level of knowledge required. This includes synergistic or additive toxicity of different alkyllead species occurring in the same spatial environment.

7.3. Toxicity to Algae

The only major comparative works on the effect of organoleads on aquatic species other than bivalves concern unicellular algae[51,113,135,149] or bacteria and fungi[142]. An extensive study of the effect of chain length upon the trialkyllead toxicity to several bacteria and fungi[142] found that toxicity increased with chain length from methyl to butyl/pentyl groups. However, longer chains from pentyl to octyl showed a marked falloff in toxicity across the whole range of eight organisms tested.

Röderer[135] also found that the toxicity of ethyl derivatives was greater than the respective methyl compounds. The data from the present study, gained from Poterioochromonas exposure is generally in full support of this work, including the absence of TEL induced toxicity. The total absence of toxicity in TEL exposed cultures found by Röderer could not be repeated in our studies because of the omnipresent Et_3Pb^+ breakdown product. Röderer's observations are surprising in this instance since we know from private communication with other workers in this area, that the washing technique used by Röderer does not produce 100% pure TEL and that is certainly what we ourselves observed.

From analyses of Poterioochromonas it appeared that Et_3Pb^+ and probably TEL are metabolised within the cell to Et_2Pb^{++}. Possibly Pb^{++} would also be ultimately involved. These findings are in apparent contrast to Röderer's suggestion that TEL is not metabolised by the algae, even though it was accumulated[113]. However this is a discrepancy in details only, the major findings for toxicity are in agreement.

In contrast to this, earlier work by several authors has been in favour of high TAL toxicity. Silverberg et al.[149] claim a 4 hour EC_{50} of <0.3 mg l^{-1} for the fresh water alga Ankistrodesmus folcatus exposed to 'biologically generated' TML. They also give values for the reductions in photosynthesis and growth as compared to control cultures for two other species, Scenedesmus quadricauda and Chlorella pyrenoidosa. These data also suggest significant TML toxicity.

Maddock and Taylor[51] suggest that TEL and Et_3PbCl have similar toxicities to Phaeodactylum tricornutum, as do TML and Me_3PbCl, the respective ethyl derivatives being more potent. In this case the similar data for particular alkyl chains might be due to TAL decomposition products present in the TAL dosed cultures causing similar toxicity to that of pure trialkyllead compounds. This possibility is not ruled out by the

authors[127].

Our results show that any toxic action in TEL or TML exposed cultures could be due to the presence of the respective trialkyllead breakdown products. Unfortunately details of relevant analyses of media used by previous workers in TAL exposure are not provided, leaving the possibility of trialkyllead action as in the case of Maddock and Taylor.

In Chapter 6 analyses of alkyllead compounds within Poterioochromonas cells and in EVT media revealed some interesting data. Cultures exposed to TEL and TML in the dark generally contained far greater cellular R_3Pb^+ concentrations than cultures exposed to Et_3PbCl and Me_3PbCl only, even though concentrations of the trialkylleads in both TAL and R_3PbCl exposed media were similar.

An even more unexpected result was the similarity in toxicity of 0.1 mg l^{-1} Et_3PbCl and 62.5 µl TEL l^{-1} illuminated cultures. Concentrations of Et_3Pb^+ in TEL exposed cultures were far greater than the 0.1 mg l^{-1} found in Et_3PbCl cultures, therefore one might expect the former conditions to exhibit greater toxicity, assuming Et_3Pb^+ to be toxic. This was not the case. With the TAL exposed cultures, reductions in cell titers were no where near those expected, i.e. Et_3Pb^+ and Me_3Pb^+ were not particularly toxic to cells under TEL or TML exposure, but were highly toxic under Et_3PbCl and Me_3PbCl exposures.

Metabolism of TAL within the cell might explain the high cellular R_3Pb^+ concentrations as the breakdown of TAL could produce R_3Pb^+, however such an explanation cannot account for the apparent lack of toxicity of Et_3Pb^+ in TEL exposed, illuminated cultures. One possible interpretation of these results is that the trialkyllead ions may actually be adsorbed to or very closely associated with the TAL molecules. This may affect

the chemistry of the ions, creating a less polar complex overall, but equally importantly preventing the ions from interacting as free molecules. This would help to explain the large variation in, for example, Et_3Pb^+ and Et_2Pb^{++} concentrations obtained from otherwise reasonably consistent analytical techniques. When adsorbed on TEL molecules in suspension, these lower analogu s would not be freely soluble and would therefore hinder the analyses.

Comparisons of TML and TEL exposed culture titers and media concentrations from Chapter 6 indicate that the difference in toxicity observed between illuminated and dark cultures is less for TML than for TEL. Again an adsorption of the trialkyllead breakdown product can provide a possible interpretation. TEL is more stable than TML under dark conditions, i.e. TML degrades to Me_3Pb^+ to a greater extent than TEL degrades to Et_3Pb^+. Therefore, more Me_3Pb^+ will be formed over a finite period than Et_3Pb^+, which results in a smaller difference between Me_3Pb^+ and TML concentrations than between Et_3Pb^+ and TEL concentrations. As the trialkyllead ion is the most toxic form, the TML dark incubated cultures would be expected to be more toxic.

One possible mechanism of alkyllead toxicity might be the disruption of membrane function as a result of the compounds passing into the cell. An association by adsorption between R_3Pb^+ and TAL molecules may reduce the disruption by increasing the lipid solubility and therefore passage across the membrane. When only ionic lead species are present, as in the case of Et_3PbCl or Me_3PbCl exposed cultures, severe disfunction may result from the polar nature of the molecules.

Another explanation to the differences in observed toxicity might be the adsorption phenomenon only. This would not rely upon any particular site of alkyllead toxic action, as in the membrane disruption case, but

merely depend upon the removal of the toxic trialkyllead from interaction with the cell. Thus toxicty would presumably be a function of the relative amounts of TAL and R_3Pb^+ within the cell or in the aqueous phase, and the degree to which the R_3Pb^+ adsorbs onto the TAL. Of course, metabolism of alkyllead compounds (dealkylation) within the cell must be closely allied to the causes or prevention of toxicity. An insight into the fate of alkyllead species within unicells would be most useful in helping to produce a more substantial explanation of the data obtained in our studies.

Many of the other algal studies mentioned above used the ^{14}C uptake method to determine the effect of alkyllead on photosynthetic activity, i.e. growth. This is an excellent parameter to monitor as it gives an indication of the general population growth in a short exposure period. Cell counts can also be most useful as long as accurate estimates of density are possible. This can prove tedious especially when optical measurements proportional to cell titers are not possible. Of course dead cells may create inaccuracies if the cells do not rapidly decompose.

It should be noted that these algal assays are not true measures of acute toxicity. Many generation times are possible in exposures lasting several days. The parameters measured are therefore a combination of both cell death and reproduction. The lack of any significant ameliorative effect on alkyllead toxicity by selenium found for Phaeodactylum was of some interest. It would be most useful to establish whether such a lack of protection to organometalics is observed in other organisms including higher beings such as vertebrates. As previously discussed, if the alleviation of toxicity by selenite action depends upon relocation of toxic materials to non-sensitive areas, then highly differentiated organisms in the presence of organometalics would be more likely to respond to the presence of selenite than a unicellular alga.

The toxicities of organotin compounds similar to those discussed and studied in this thesis, have been determined for various algal species[150]. This is useful in as much as the general trends of alkyltin toxicity can be compared with those for alkyllead. As might be expected, the toxicity of alkyltin compounds is predominantly greater than that of organic tin compounds. This is also true of alkyllead and inorganic lead compounds. From the several alkyl species studied (methyl to octyl), the trialkyltins were most toxic, a trend similar to our alkyllead findings. Equally interesting was the increasing toxicity associated with increasing carbon chain length for any given series (eg. trialkyltin). Again these observations match the respective alkyllead toxicities.

Thus, in terms of this single study, alkyltin compounds appear to possess very similar toxicity patterns to alkyllead compounds when algae are used as the bioassay system. A more general literature suggests that tetraalkyl and trialkyl tin compounds are usually more toxic than the respective dialkyl and monoalkyl tins.

Wong et al.[150] also investigated the relationship between particular alkyltins and the n-octanol/water partition coefficients for these compounds. This coefficient is commonly used to estimate the lipid solubility and toxicity of organic materials. A positive relationship was found for the trialkyltin compounds but other types were less well correlated. In other words the greater the partitioning of trialkyltin into the n-octanol layer, the greater the toxicity. It would be interesting to compare alkyltin partition coefficients vs. toxicity with those for alkyllead, however at present there is a lack of such data for alkyllead compounds.

7.4 Comparisons With Other Literature And Analytical Considerations.

Our observations, for the most part, on alkyllead toxicity are in agreement with those of other authors, i.e. ethyl compounds are more toxic

than the corresponding methyl analogues, to the majority of the spectrum of organisms tested. Of course as in all facets of biology, there are exceptions to this such as Mytilus (trialkyllead)[51] and mice[151] and dogs[41] (TAL). The other major trend is that trialkyls are generally more toxic than dialkyls or inorganic lead[27].

It is interesting to consider the absence of TAL toxicity in this study with the same findings documented in several of the mammalian investigations. Cremer[37] suggests that the toxicity of TEL is due to its rapid metabolism by particular organs (in rats) to Et_3Pb^+. This ionic species is then the cause of toxicity. The liver microsomes appear to catalyse the enzymatic dealkylation to the greatest extent. Similarly, metabolism of TEL to Et_3Pb^+ is most likely in the alga Poterioochromonas as shown by the studies described in section 6.7. A lack of TEL toxicity in its pure form is thus indicated by two vastly different organisms, both being sensitive to Et_3Pb^+ at relatively low concentrations.

Acute toxicity data for aquatic organisms, excluding that discussed above, are scant. TAL toxicity to a few fish species has been investigated by various authors as can be seen in Table 7.1.

TABLE 7.1 TOXICITY OF TAL TO FISH

Species	Parameter	Conc.	Compound	Ref.
Rainbow trout	96 hr LC 50		TML	152
Bluegill sunfish	48 hr LC 50	1·4	TEL	153
" "	24 hr LC 50	2·0	TEL	153
Plaice	96 hr LC50	0·23	TEL	51
"	"	0·05	TML	51
Unknown	48 hr LC50	0·1	TML-CB	50
"	"	0·065	TEL-CB	50

The greater TEL toxicity reported for the majority of studies is not apparent from these studies, with one study for the trend and one against. However since analyses were not carried out, the toxic agent cannot be identified. The fact that such toxic activity was recorded for TAL appears to contradict the idea of non-toxicity put forward in this and the other studies[37,113].

It is difficult in many cases, especially with older papers, to discover the exact dosing procedure and whether adequate analytical techniques were applied to the toxicity bioassays. It is clear that those aspects are of prime concern if toxicity is to be attributed to any single alkyllead compound. This is perhaps of lesser importance in trialkyl or dialkyl lead salt assays, as they are relatively stable, but for TAL it is all too easy to include the effects of the lower analogue breakdown products. Assays conducted under illumination would be particularly subject to such contamination.

In the present study steady state concentrations of any of the alkyllead compounds tested were difficult to achieve, as illustrated by the analytical data variability. Theoretical concentrations calculated prior to dosing and not backed up by analyses of the dose containing medium are therefore open to severe criticism. Several reports of TAL toxicity appear to fall within this category. Even when analyses are performed, the generated data may not be particularly accurate. When specific analyses for individual alkyllead species are used, accuracy may be low because of the often complex nature of the analytical methods. Although sophisticated technology is available and capable of speciating quantitatively single alkyllead compounds, the usually low level concentrations in samples make contamination, and consequently inaccurate determinations, possible. Clean laboratories and pristine apparatus contribute to the reduction of contamination sources, but in some circumstances the sample itself may be the problem. For example,

to determine the concentration of TAL in solution a sample must be taken from some region within the medium. In our experience it is often the case for TAL to form a fine layer at the surface of the medium, due to surface tension. If such a surface layer is present at the time of sampling, it is difficult to withdraw an aliquot without contaminating the sampling device (e.g. automatic pipette). The TAL may then be transferred to the preconcentrated solvents for analysis, resulting in incorrect evaluations.

The analytical technique applied to a sample must also be given some thought. For example, analyses of TEL exposed Poterioochromonas cells performed by nitric acid/bromine oxidation[113] provides total alkyllead determinations. From the data obtained it was concluded that TEL had been accumulated by the cells but not metabolised. Such a conclusion cannot be drawn from an analytical technique measuring total lead content. Any number of different ethyllead species present would not be identified, apart from that they contained lead, by this procedure.

Optimally then, several different analytical techniques could be used from the large variety available, to give an estimation without any unseen bias arising from individual techniques. However, this is far from practical in terms of time involved, volumes of samples required and instrumentation needed, and often determination by one method only is possible.

Our own analyses for the alkyllead compounds used provide a basis for comparison with other works, of the relative stabilities of the compounds in aqueous media. These data, mainly emanating from analyses of EVT medium used for exposing Poterioochromonas, follow some results of organolead decomposition that are generally accepted in the literature[6,5]. These are that methyl compounds are more reactive in the dark than ethyl

leads but the opposite applies under illuminated conditions.

7.5. Relevance to the Environment

The release of alkyllead compounds into the atmosphere with exhaust discharges from automobiles results in generally widespread, low level alkyllead pollution, but it is unlikely that this causes significant short or long term damage to the estuarine environment. By the time surface runoff and atmospheric deposition reaches coastal systems, most of the organic lead present will have either decomposed to inorganic lead or become adsorbed onto particulates or complexed to naturally occurring chemical species. Biological availability of the organolead might then be low. However, it should be noted here that as both Scrobicularia and Macoma are preferentially deposit feeders, they might well absorb alkyllead compounds adsorbed onto sediments. It is not known whether once ingested alkyllead would become desorbed and accumulate in the animals' tissues.

Alkylation of lead (inorganic) and in particular methylation, is thought to be a likely source of biologically[20,21,154] or chemically[24,25] generated alkyllead in sediments, presumably including estuaries. There are abundant data to suggest that true biological methylation of Pb^{++} does not occur[24, 25, 26] and that chemical alkylation would result in only low level production with little environmental impact[25].

Significant acute or chronic toxicity of alkyllead compounds is therefore most likely to originate from loss at sea of neat compound or through ship collisions and wreckings, or by accidental discharge in industrial alkyllead manufacturing plant effluent. Such effluents are strictly controlled in Britain to ensure minimal alkyllead content, both at source and by monitoring of environmental levels in the proximity of outfalls. Such monitoring may be undertaken by a regional water authority and by

the manufacturers themselves. Severe pollution is now unlikely via this source.

As previously discussed, shipping accidents such as the Cavtat incident[155] may occur albeit infrequently. However, it is often the case that commercial TAL compound is transported in barrels, as with the Cavtat. In instances of compound loss therefore, recovery of the majority of the cargo is possible, especially in shallower or coastal locations. Thus, severe pollution could again be most probably avoided.

In general then, use of alkyllead compounds as commercial antiknocking agents is not likely to result in widespread and severe organolead pollution. High level acute contamination is only possible at present through major loss during transportation, or in the event of high alkyllead concentrations in industrial discharges.

The reasons why some compounds are toxic to an organism while others are not, and how compounds may be toxic to some organisms but not to others, form a highly complex subject far beyond the scope of this study. An understanding of such a subject, i.e. structure-activity relationships, would help to explain why TEL may be more toxic than TML (if at all), but less toxic to Et_3Pb^+.

To claim that TEL is non-toxic to Scrobicularia, Phaeodactylum and Poterioochromonas, from the work done in this study, would be exceeding the limits of the data. Under the limted environmental conditions, concentrations and techniques used, TEL appears to exert a minimal toxicity as indicated by the criteria chosen. To extend these findings, without qualification, would be unjustified. However, care should be taken when considering such restrictions so as to avoid excessively limiting the data. For example, the lack of TEL toxicity to Poterioochromonas at 26°C and other specific conditions in this study, would most likely be

true at other temperatures and conditions, although there are no data to support this here.

We conclude that alkyllead compounds are generally highly toxic to bivalve molluscs and unicellular algae with the exception of TAL. However this exception may be of little importance in the marine or freshwater environment where pure TAL occurrence would be highly improbable. The vast diversity of substances occurring naturally, or through man's impact, in these systems would most certainly bring about decomposition of TAL to the lower toxic analogues.

The life time of alkyllead species in aquatic environments is highly dependant upon that particular environment as well as the species itself. For example, triethyllead would probably be more stable in a deep freshwater lake, below the limits of light penetration, than in the surface waters of a coastal marine system. Abiotic factors influencing the physical and chemical nature of alkyllead compounds will therefore also affect the biological availability and toxicological properties. For estuarine systems where, at present, alkyllead concentrations are generally likely to be very low, the danger to organisms is probably insignificant. However, in areas where human activity results in measureable concentrations of say less than 0.1 mg l^{-1}, severe biological consequences may result. These may take the form of bioaccumulation in bivalves, algae and probably other organisms or even acute lethal toxicity.

Alkyllead present in the estuarine environment would be expected to have a relatively short residence time when it is biologically available. Photolysis, catalysis, adsorption or other processes rapidly act to reduce TAL concentrations, with trialkyl and dialkyl lead compounds lasting some-what longer. The influences on alkyllead generally would

bring about an overall dealkylation to inorganic lead. With the probable advent of lead-free petrol in this country at some time before the end of the century[11], and in many others also, the global pollution of alkyllead can only become less and eventually insignificant.

EXPERIMENTAL SECTION

EXPERIMENTAL

E.1. General

All reagents and solvents used throughout this study were of Analar grade.

Artificial seawater was made up according to the method of Lyman and Fleming[94], and consisted of the following weights (g) of salts per 10 l:

NaCl	240.0
$MgCl_2$	50.8
Na_2SO_4	39.9
$CaCl_2 2H_2O$	14.9
KCl	6.6
$NaHCO_3$	1.96
KBr	0.98
H_3BO_3	0.27
$SrCl_2$	0.24
NaF	0.03

Transfer and dilution of alkyllead solutions was effected with variable volume automatic pipettes ('Finnpipette') 5 - 55 µl and 1 cm^3 - 5 cm^3 used in conjunction with disposable tips.

Most alkyllead compounds were kindly donated by the Associated Octel Company Ltd., including Me_3PbCl, Me_2PbCl_2, TML (pure), Et_3PbCl, Et_2PbCl_2 and TEL (pure). All alkyllead salts were 98% pure. Pure TAL refers to TAL in absence of any solvents but probably includes TAL decomposition products. In addition GLC-MS, ASV and AAS facilities were provided by A.O.C.Ltd. All GLC-MS and some polarographic analyses were performed by A.O.C.Ltd. personnel.

All glass apparatus were deleaded by soaking in concentrated nitric

acid for several hours prior to use, followed by thorough rinsing with distilled water.

E.2.1. Instrumentation

Atomic absorption spectrophotometers employed were:

1. Perkin Elmer PE 303 with a hollow cathode lead lamp at 283.3 nm, a Perkin Elmer heated graphite atomizer HGA-70, an automatic null recorder readout PE 303-0103 and an automatic type 5211-11 chart recorder.
2. Perkin Elmer PE 560 with lamp as above and a Perkin Elmer programmer HGA 500.

Quantitative determinations of TAL contained in hexane or toluene from aqueous or biological sample extractions respectively were by a 12-12 F quadrupole mass spectrometer preceded by either of two GLC columns. A VG 2000 Datasystem was employed to calculate and printout results. Hexane samples were passed through a capillary column 50 m by 0.3 mm internal diameter, packed with OV-1. Toluene samples were passed through a stainless steel packed column 2 m packed with 5% carbowax 20M.

Operating conditions for both types of samples were as follows:

columns heated by oven

injection temperature = 40 °C

column temperature = 70 °C rising to 150 °C

source temperature = 200 °C

carrier gas was helium at a flow of 30 cm^3 min^{-1}

retention time for TML was approximately 5 minutes, and for TEL was 6.5 minutes.

Polarographic analyses were performed with a Princeton Applied Research 174A polarographic Analyser with 315A programmer.

Liquid scintillation counting was performed with a Beckman LS 7500 microprocessor controlled liquid scintillation counter with γ-source

internal standardisation.

Ultraviolet and visible spectra were recorded on a Pye-Unicam high resolution spectrophotometer.

pH measurements were recorded with a Uniprobe model 301 laboratory pH meter.

Dissolved oxygen recordings were made with a Griffin portable, battery powered D.O. meter.

Light intensity was recorded with an Evans electroselenium Ltd. lightmaster.

E.2.2. Analysis of Aqueous Samples

E.2.2.1. Dithizone/AAS

The following solutions were required:

1.0M Iodine Monochloride

27.75 g KI was dissolved in about 100 cm^3 glass distilled water in a 1 litre beaker. 111.2 cm^3 concentrated HCl was added slowly, allowing the mixture to reach room temperature. 18.75 g KIO_3 was added gradually, whilst stirring, until all of the free iodine had re-dissolved giving a clear orange-red solution. The solution was diluted to 250 cm^3 with distilled water, and stored in the dark.

Buffer Solution

The buffer solution was made up from sodium sulphite, ammonium citrate and concentrated ammonia solutions, prepared as follows:

125g anhydrous sodium sulphite in 1 litre distilled water. This solution was deleaded by extraction with dithizone solution until no further colour change was noted in the dithizone.

400 g citric acid monohydrate was moistened with distilled water in a 2 litre beaker and O-cresol red indicator solution added. Concentrated ammonia solution (S.G. = 0.88) was added slowly until the solution was just alkaline. Distilled water was added to 1 litre. This solution was also deleaded with dithizone solution.

1 litre of buffer was made up by mixing:

20 cm^3 ammonium citrate solution
160 cm^3 sodium sulphite solution
160 cm^3 concentrated ammonia solution (S8 0.88)
660 cm^3 distilled water.

0.2M EDTA Solution

37.2 g ethylene diamine tetraacetic acid disodium salt was dissolved in 320 cm^3 distilled water with heating if required. The solution was transferred to a 500 cm^3 volumetric flask and diluted to the mark.

Dithizone Solution

5 mg dithizone (Eastman Kodak) was dissolved in 250 cm^3 CCl_4.

Nitric Acid/Hydrogen Peroxide Solution

1 cm^3 Aristar concentrated nitric acid was added to 50 cm^3 distilled water. 1 cm^3 Analak 100-volume hydrogen peroxide was then added and diluted to 100 cm^3 with distilled water.

Method:

10 cm^3 of a sample was added to a blackened 150 cm^3 separating funnel. 1.5 cm^3 ICl solution was introduced and made up to 15.0 cm^3 with distilled water. 20 cm^3 buffer solution was added and rapidly swirled to mix, reducing any free iodine formed. 3.5 cm^3 EDTA solution was added to complex any inorganic lead present, followed by 5 cm^3 dithizone solution. The funnel was shaken for 30 seconds

and the layers allowed to separate. The lower organic layer was run off into a PTFE tube. 5 cm^3 CCl_4 was added to the funnel and shaken for a further 30 seconds. After separation this too was run off into the PTFE tube. The organolead was then back-extracted into 2 cm^3 of the acid/peroxide solution which was added to the tube and shaken for 60 seconds. After separation the acid layer was analysed by AAS by injecting 20 µl samples into the furnace using the following programme:

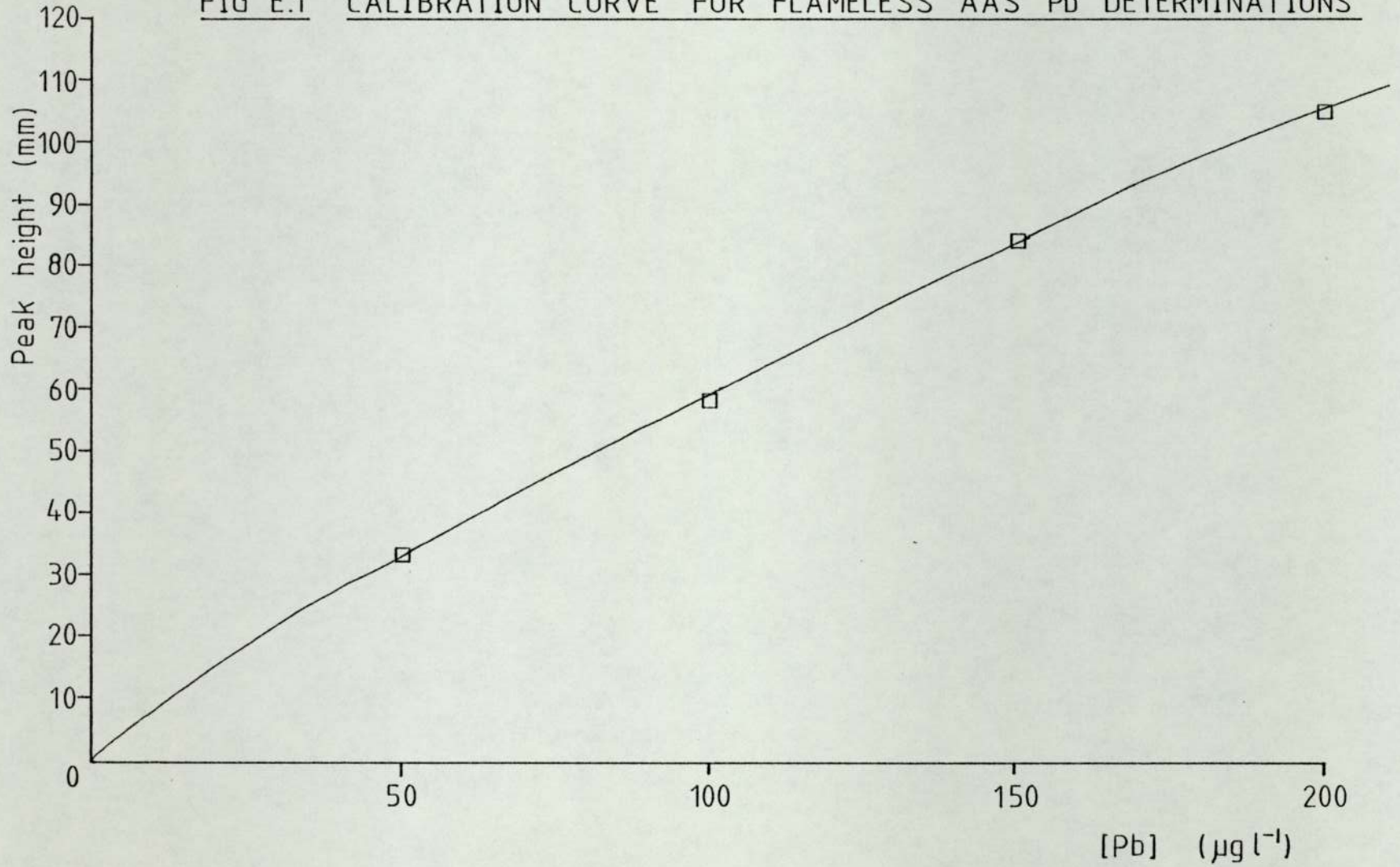
drying	30 seconds at 100°C
ashing	60 seconds at 490°C
atomisation	5 seconds at 2350°C

This method estimates total alkyllead content. If TAL was suspected in the sample, the initial 10 cm^3 sample was shaken with 5 cm^3 n-hexane for 30 seconds and allowed to separate. The lower aqueous phase was then run off and analysed for trialkyl and dialkyl lead as above, or by polarographic determination. The hexane layer was analysed by GLC-MS or by the above procedure. In this latter case the hexane was removed prior to dithizone addition.

Calibration was achieved by plotting the absorbance values of standard additions of alkyllead stock solutions extracted by the above procedure. 0, 100, 200, 300 and 400 µl of a 1.0 mg l^{-1} standard solution were normally used. For every analysis, all 5 standards were run to avoid deviation in day to day instrument performance. Fresh standards were used when new batches of reagent were made up.

Using this method the normal range was 0.003 - 0.2 mg l^{-1} organic lead. If any sample lay outside of this range, a further sample was diluted and then extracted.

FIG E.1 CALIBRATION CURVE FOR FLAMELESS AAS Pb DETERMINATIONS



E.2.2.2. PAR

1.0M Iodine Monochloride

As prepared for the dithizone extraction procedure.

Buffer Solution pH 9.0

60 g sodium sulphite hydrated ($Na_2SO_3.7H_2O$) and 46 g tri-ammonium citrate ($(NH_4)_3C_6H_5O_7$) were dissolved in 600 cm^3 distilled water, in a 1 litre flask. 5 g potassium cyanide (KCN) was dissolved in about 50 cm^3 distilled water and 100 cm^3 ammonia solution (SG 0.88) was added to this and the whole was diluted to volume with distilled water.

0.2M CDTA Solution

14.55 g 1,2- Diaminocyclohexane-NNN'N'-tetra-acetic acid (CDTA) was dissolved in 100 cm^3 1M sodium hydroxide (NaOH). This was then diluted to the mark with distilled water in a 200 cm^3 volumetric flask.

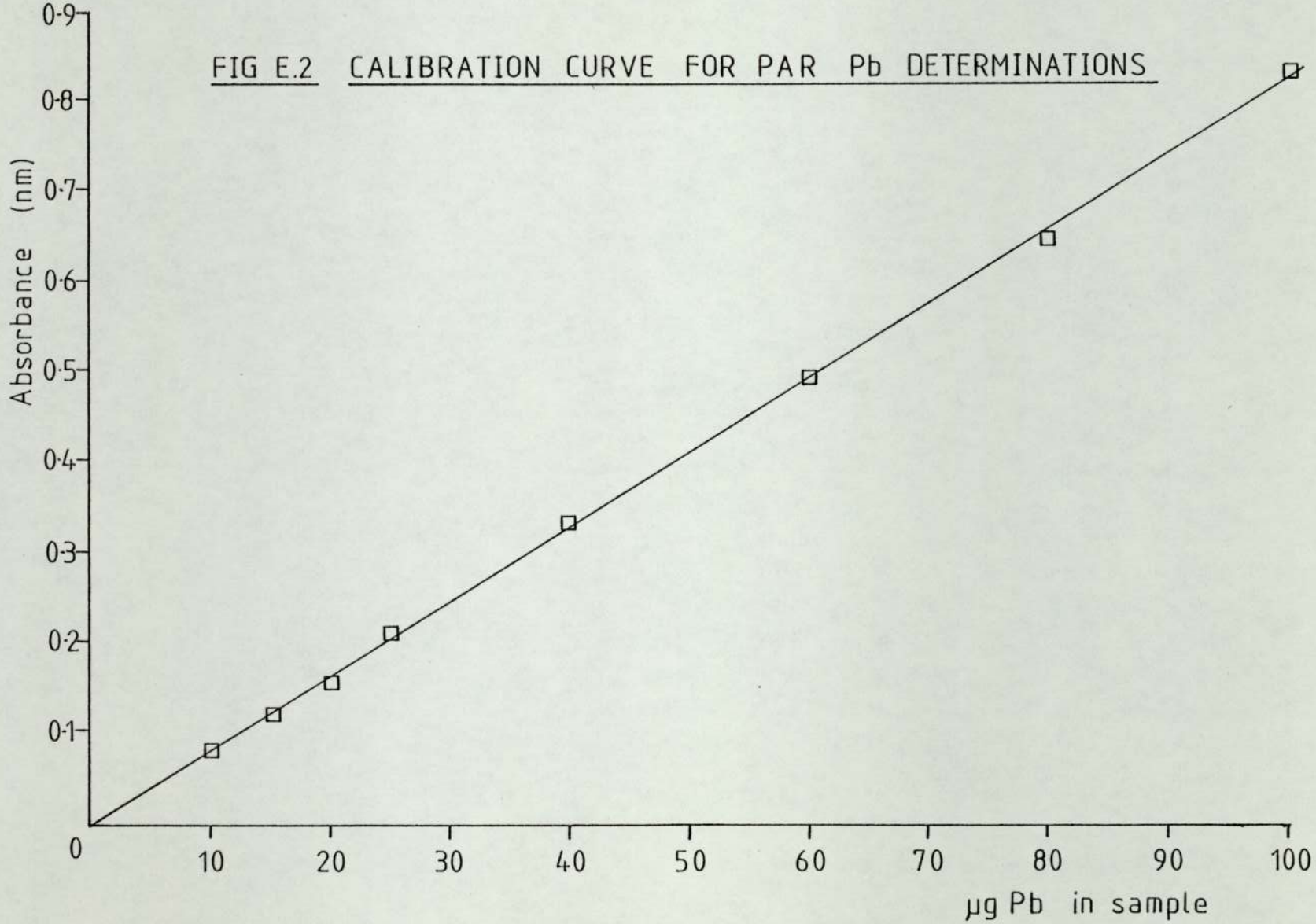
PAR Solution

0.235 g PAR sodium salt (Hopkin and Williams) was dissolved in about 200 cm^3 distilled water. The solution was transferred to a 1000 cm^3 volumetric flask and diluted to volume.

Method:

A sample up to a maximum volume of 10 cm^3 was added to a de-leaded, blacked-out 25 cm^3 volumetric flask. 1 cm^3 ICl solution was pipetted into the flask, followed by 5 cm^3 distilled water. After standing for 5 minutes, 5 cm^3 buffer solution was added and the flask swirled to mix. Again this dissolved any free iodine that may have formed. 1 cm^3 CDTA and 3 cm^3 PAR solutions were added and diluted to volume with distilled water. The flask was then shaken to mix, after which absorbance was determined at 515 nm against a blank. A calibration curve was employed to estimate the amount of the particular alkyllead present in the sample.

FIG E.2 CALIBRATION CURVE FOR PAR Pb DETERMINATIONS



Because the PAR analytical procedure was used only infrequently, a new calibration curve was plotted at each analysis.

E.2.2.3. Polarographic Determination

The following reagents are required:

Sodium Acetate Buffer, pH 5.0

26 g anhydrous sodium acetate was dissolved in about 80 cm^3 distilled water, in a 100 cm^3 volumetric flask. 5 cm^3 glacial acetic acid was added and diluted to volume.

Mercuric Chloride Solution

0.135 g $HgCl_2$ was dissolved in 100 cm^3 distilled water. This gives 1 mg Hg cm^3.

CDTA 0.2M

This should be 0.2M with respect to CDTA and 0.5M with respect to NaOH.

Electrolyte Solution

This should be 0.51 Molar with respect to NaCl and 0.07 Molar with respect to $MgCl_2$.

Sodium Hydroxide Solution

8.0 gm NaOH was dissolved in 100 cm^3 distilled water.

Stock Trialkyllead Solution

0.159 g Et_3PbCl or 0.139 g Me_3PbCl was dissolved in 200 cm^3 distilled water, transferred to a 1 litre volumetric flask and diluted to the mark.

Stock Dialkyllead Solution

0.162 g Et_2PbCl_2 or 0.149 g Me_2PbCl_2 was dissolved in 100 cm^3 distilled

water, as for trialkyllead stocks.

Trialkyllead and Dialkyllead Standards

These are diluted stock solutions giving a final concentration of 1 mg Pb l^{-1}.

Anodic Stripping Cell

The cell comprises a platinum spiral counter electrode in a salt bridge, a saturated calomel reference electrode in a salt bridge and a glassy carbon electrode (3 mm disc). The electrode is mixed by a PTFE conically perforated disc vibrated by an aquarium air pump (Rena 301).

Purging nitrogen was passed through a purification train consisting of a de-oxygenating catalyst, an active carbon scrubber and a 0.8 nm pore membrane filter. Flow is 20 cm^3 min^{-1}.

Method:

The electrode (glassy carbon) was initially conditioned in an ultrasonic bath for 20 seconds. It was then washed in distilled water and positioned above the mixer in the cell. 50 cm^3 electrolyte was poured into the cell with 0.5 cm^3 acetate buffer, $HgCl_2$ solution and CDTA solution. The deposition potential was -1.0 V and the cell was purged for 5 minutes prior to blank determination. A suitable aliquot of the sample (< 2 cm^3) was then introduced into the cell. Deposition potential was set at -0.70 V and the purge time was one minute. During the run a peak at -0.52 V indicated presence of dialkyllead. Repeating the run from -1.0 V gave a trialkyllead peak at -0.70 V.

Calibration of the peaks was achieved by adding 20 µl standard alkyllead solution by automatic micropipette and repetition of the scans. Results were calculated by the polarograph computor and displayed on the VDU as ppb values.

E.2.3. Analysis of Biological Samples

The following reagents are required:

Lead nitrate

Sodium chloride

Potassium iodide

Sodium benzoate

EDTA 0.2M (as for dithizone procedure)

Nitric acid 1% m/v

Toluene

Method:

All biological tissues were analysed by the same procedure.

A known wet weight of tissue (about 1 g for molluscs) or a known number of algal cells were placed in a homogenisation tube, containing 15 cm^3 distilled water. 0.2 g $PbNO_3$ was added and homogenised for 30 seconds after which 3.5 cm^3 0.2M EDTA was added and shaken. 6 g NaCl, 1 g KI, 2 g Na benzoate and 10 cm^3 toluene were then added and homogenised for a further 2 minutes. The mixture was transferred to a centrifuge tube and spun at 4000 rpm for 15 minutes. 5 cm^3 of the toluene was removed and transferred to a 50 cm^3 separating funnel, where it was shaken for one minute with 10 cm^3 1% nitric acid. After separation the lower aqueous phase was run off and determined by ASV, or re-extracted using the dithizone/AAS procedure. The latter technique is obviously inferior to polarographic analysis.

A further toluene aliquot was removed for TAL analysis by GLC-MS or by the dithizone/AAS procedure. Again the former technique was greatly preferred.

E.3.1. Condition Factors

Condition factors for Macoma balthica and Scrobicularia plana were calculated on the basis of percentage flesh weight to total body weight. Although wet weight measurements are subject to greater inaccuracy than dry weight, the latter do not provide any information as to the changes in liquid content of the animal.

$$\text{Condition Factor} = \frac{\text{wet flesh weight}}{\text{wet total weight}} \times 100$$

Table E.1. Condition Factors for Control Animals Under Static Renewal Test Conditions

	Scrobicularia					Macoma				
Time /d	1	2	3	$\bar{x}$	$\pm$ 1S.D.	1	2	3	$\bar{x}$	$\pm$ 1S.D.
0	45.0	55.0	48.5	49.5	5.2	32.5	43.3	49.6	41.8	8.6
4	58.5	29.4	40.0	42.6	14.7	43.4	36.1	37.3	38.9	3.9
10	22.5	32.6	35.8	30.8	6.8	41.8	28.1	37.3	35.5	7.7
15	30.5	22.3	27.0	26.5	4.3	44.5	46.1	33.8	41.6	7.3
17	26.4	37.9	24.9	29.8	7.1	36.9	29.8	35.5	34.0	3.8
24	28.3	25.4	38.1	30.6	6.6	35.5	24.8	32.0	30.7	5.4
28	40.1	26.6	51.0	39.2	12.9	42.9	16.1	52.9	37.3	20.2
31	27.6	41.9	24.0	31.1	10.6	29.4	35.6	33.0	32.6	30.0
38	35.4	26.2	21.8	27.9	6.7	53.9	30.3	45.1	43.1	11.8
41	39.7	39.5	24.7	35.0	8.2	18.5	37.7	32.6	29.6	9.9
45	23.5	39.9	34.8	32.8	9.0	26.3	21.5	33.9	27.3	6.3
52	29.1	21.4	34.5	28.3	6.3	31.1	27.2	39.8	32.8	6.4
59	25.6	17.8	33.7	25.8	8.0	26.9	24.7	38.4	30.1	7.4

E.2. Wet Weights (g) of a) Scrobicularia and b) Macoma Controls Under Static Renewal Tests

a) Scrobicularia

Time	1		2		3	
(days)	soft	total	soft	total	soft	total
0	2.97	6.60	2.68	4.87	2.34	4.82
4	3.31	5.66	1.52	5.17	2.02	5.05
10	1.30	5.78	2.04	6.26	1.79	5.00
15	1.49	4.89	1.36	6.10	1.48	5.48
17	1.00	3.79	2.17	5.73	1.25	5.02
24	1.35	4.77	1.41	5.55	1.87	4.91
28	2.26	5.64	1.56	5.86	2.88	5.65
31	1.28	4.64	2.70	6.44	1.30	5.42
38	1.83	5.17	1.09	4.16	1.19	5.46
41	2.04	5.14	2.51	6.35	1.21	4.90
45	1.11	4.72	2.39	5.99	1.55	4.45
52	1.34	4.60	1.10	5.14	1.55	4.49
59	1.22	4.77	0.89	5.00	1.66	4.93

b) Macoma

0	0.22	0.68	0.26	0.60	0.28	0.56
4	0.26	0.60	0.24	0.66	0.26	0.69
10	0.24	0.57	0.20	0.71	0.26	0.70
15	0.27	0.61	0.47	1.02	0.24	0.71
17	0.25	0.68	0.21	0.70	0.25	0.70
24	0.27	0.76	0.18	0.73	0.23	0.72
28	0.20	0.47	0.06	0.37	0.31	0.59
31	0.21	0.71	0.23	0.65	0.23	0.70
38	0.29	0.54	0.20	0.66	0.34	0.75
41	0.08	0.43	0.25	0.66	0.22	0.67
45	0.27	1.03	0.19	0.88	0.23	0.68
52	0.22	0.71	0.20	0.74	0.25	0.63
59	0.13	0.48	0.16	0.65	0.25	0.65

Table E.3a. Wet Weights and Condition Factors of Scrobicularia at the Start (S) and Finish (F) of Exposure to Various Alkyllead Compounds

Compound tested		Weights (g) and Condition Factor (% soft ÷ total)														
		soft	total	CF	soft	total	CF	soft	total	CF	soft	total	CF	soft	total	CF
Control	S	2.97	6.60	45.0	2.68	4.87	55.0	2.34	4.82	48.5						
	F	1.22	4.77	25.6	0.89	5.00	17.8	1.66	4.93	33.7						
36µl TEL l^{-1}	S	2.99	6.57	45.5	1.51	4.45	33.9	1.83	4.84	37.8	1.66	4.65	35.7	2.26	4.82	46.9
	F	1.08	5.12	21.1	0.88	5.15	17.1	1.07	5.27	20.3	1.75	4.89	35.8	1.15	5.00	23.0
72µl TEL l^{-1}	S	2.51	5.21	48.2	1.69	4.58	36.9	1.84	4.83	38.1	1.78	4.89	36.4	2.40	4.97	48.3
	F	1.90	4.77	39.8	1.48	5.00	29.6	1.15	5.20	22.1	1.27	5.34	23.8	1.21	4.22	28.7
Me_3PbCl	S	2.48	5.05	49.1	2.30	5.09	45.2	1.57	4.95	31.7	2.33	6.15	37.9	2.83	6.72	42.1
	F	1.32	4.14	31.9	0.98	4.47	21.9	2.41	5.96	40.4	0.98	4.90	20.0	1.34	4.39	30.5
Et_3PbCl	S	2.76	6.11	45.2	2.21	5.07	43.6	2.47	4.95	49.9	2.81	5.83	48.2	1.75	4.81	36.4
	F	2.79	5.86	47.6	2.21	5.15	42.9	2.61	6.38	40.9	1.49	4.50	33.1	1.70	4.72	36.0

Table E.3b. Wet Weights and Condition Factors of Macoma at the Start (S) and Finish (F) of Exposure to Various Alkyllead Compounds

Compound tested		Weights (g) and Condition Factor (% soft ÷ total)														
		soft	total	CF	soft	total	CF	soft	total	CF	soft	total	CF	soft	total	CF
Control	S	0.22	0.68	32.5	0.26	0.60	43.3	0.28	0.56	49.6						
	F	0.13	0.48	26.9	0.16	0.65	24.7	o.25	0.65	38.4						
36µl TEL l^{-1}	S	0.28	0.63	44.3	0.31	0.63	49.6	0.14	0.53	26.5	0.28	0.77	36.6	0.28	0.72	38.9
	F	0.20	0.68	29.6	0.32	1.12	28.5	0.16	0.70	23.0	0.27	0.70	38.3	0.25	0.68	36.7
72µl TEL l^{-1}	S	0.25	0.63	39.5	0.18	0.69	25.9	0.23	0.61	37.4	0.29	0.63	45.8	0.30	0.69	43.5
	F	0.15	0.68	22.1	0.24	0.70	34.3	0.19	0.71	26.6	0.17	0.73	23.3	0.23	0.71	32.6
Me_3PbCl	S	0.29	0.62	46.7	0.22	0.67	32.8	0.26	0.76	34.3	0.26	0.62	41.7	0.27	0.63	42.6
	F	0.14	0.59	23.5	0.28	0.73	38.5	0.12	0.66	18.3	0.14	0.66	21.1	0.15	0.59	25.6
Et_3PbCl	S	0.32	0.63	50.7	0.31	0.64	48.6	0.28	0.61	45.8	0.24	0.73	33.1	0.23	0.58	39.8
	F	0.31	0.64	48.8	0.28	0.61	45.7	0.15	0.57	26.5	0.33	0.65	51.1	0.30	0.64	46.9

E.3.2. Concentration of Me_3Pb^+ over Natural Sediment

Natural sediment was not used in toxicity tests for several reasons, one of which was the fall in an aqueous concentration of 1 mg l^{-1} Me_3PbCl resulting from rapid adsorption onto the sediment.

Table E.4. Decreasing Me_3Pb^+ Concentration (mg l^{-1}) over Natural Sediment

Time (hrs)	Seawater ± 1 S.D. $[Me_3Pb^+] + [Me_2Pb^{++}]$
0	1.05 ± 0.02
1	0.64 ± 0.18
2	0.69 ± 0.10
24	0.58 ± 0.12
50	0.48 ± 0.14
76	0.34 ± 0.06
170	0.21 ± 0.05
194	0.24 ± 0.08
242	0.18 ± 0.02

E.3.3. Statistical Differences in Replicate Alkyllead Concentrations

Replicate concentrations were analysed using the Student's t-test to determine any statistically significant differences. The following t values relate to Tables 3.2, 3.3 and 3.4.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \qquad \text{where} \qquad s^2 = \frac{f_1 s_1^2 + f_2 s_2^2}{f_1 + f_2}$$

Table 3.2. (N.S. = not significant. S = significant)

0.05 mg l^{-1} Et_3PbCl	t = 0.35	f = 7	P > 0.5	N.S.
0.10 mg l^{-1} Et_3PbCl	t = 0.17	f = 6	P > 0.5	N.S.
0.50 mg l^{-1} Et_3PbCl	t = 1.8	f = 6	0.05 > P > 0.025	S.

Table 3.3.

0.05 mg l^{-1} Et_3PbCl	t = 0.78	f = 8	0.5 > P > 0.2	N.S.
0.10 mg l^{-1} Et_3PbCl	t = 0.26	f = 6	P > 0.5	N.S.
0.50 mg l^{-1} Et_3PbCl	t = 0.85	f = 4	0.5 > P > 0.2	N.S.

Table 3.4.

0.05 mg l^{-1} Me_3PbCl	t = 0.26	f = 7	P > 0.5	N.S.
0.10 mg l^{-1} Me_3PbCl	t = 0.61	f = 7	P > 0.5	N.S.
0.50 mg l^{-1} Me_3PbCl	t = 0.23	f = 4	P > 0.5	N.S.

E.3.4. Cummulative Mortalities of Bivlaves Exposed to Varying Alkyllead Compounds

The following Tables support the cummulative mortality curves plotted in Figs.3.7, 3.8, 3.9, 3.10, 3.12 and 3.13.

Table E.5. Cummulative Mortality of Scrobicularia Exposed to Me_3PbCl Concentrations Between 0.00 and 1.25 mg l^{-1}

Time (days)	% Cummulative mortality				Cummulative No. of deaths			
	0.0	0.08	0.11	1.25	0.0	0.08	0.11	1.25
3	-	-	-	10	-	-	-	2
4	-	10	-	10	-	2	-	2
14	-	10	20	10	-	2	4	2
15	-	10	20	40	-	2	4	8
16	-	10	20	55	-	2	4	11
17	-	10	20	60	-	2	4	12
18	-	10	20	70	-	2	4	14
21	-	10	20	80	-	2	4	16
24	-	10	20	95	-	2	4	19
27	-	10	30	100	-	2	6	20
28	-	20	30		-	4	6	
29	10	20	30		1	4	6	
30	10	20	50		1	4	10	
50	10	50	75		1	10	15	
60	10	50	75		1	10	15	

Table E.6. Cummulative Mortalities of Macoma Exposed to Me_3PbCl Concentrations Between 0.00 and 6.6 mg l^{-1}

Time	% Cummulative mortality						Cummulative No. of deaths					
(days)	0.00	0.04	0.12	0.44	3.2	6.6	0.00	0.04	0.12	0.44	3.2	6.6
1	-	-	-	-	-	10.0	-	-	-	-	-	1
2	-	-	-	-	-	20.0	-	-	-	-	-	2
4	-	-	-	-	-	30.0	-	-	-	-	-	3
8	3.1	-	-	-	-	50.0	1	-	-	-	-	5
9	3.1	-	9.4	-	-	70.0	1	-	3	-	-	7
10	3.1	3.1	9.4	-	-	70.0	1	1	3	-	-	7
11	3.1	3.1	9.4	-	30.0	90.0	1	1	3	-	3	9
14	3.1	3.1	9.4	-	70.0	100.0	1	1	3	-	7	10
16	3.1	3.1	18.8	37.0	90.0		1	1	6	17	9	
17	3.1	3.1	21.9	75.0	90.0		1	1	7	24	9	
18	3.1	6.3	21.9	90.6	100		1	2	7	29	10	
21	3.1	6.3	39.4	93.8			1	2	19	30		
22	3.1	6.3	59.4	100			1	2	19	32		
26	6.3	6.3	65.6				2	2	21			
31	6.3	6.3	71.9				2	2	23			
35	6.3	12.5	71.9				2	4	23			
36	6.3	18.8	90.6				2	6	29			
38	6.3	18.8	96.9				2	6	31			
39	12.5	18.8	100.0				4	6	32			
55	12.5	18.8					4	6				
60	12.5	18.8					4	6				

Table E.7. Cummulative Mortalities of Scrobicularia Exposed to Et_3PbCl Concentrations Between 0.00 and 1.08 mg l^{-1}

Time	% cummulative mortality					cummulative number of deaths				
(days)	0.00	0.14	0.24	0.94	1.08	0.00	0.14	0.24	0.94	1.08
6	-	-	-	13.3	-	-	-	-	2	-
7	-	-	6.7	13.3	13.3	-	-	2	2	2
10	-	6.7	20.0	53.3	86.0	-	2	6	8	13
11	-	6.7	26.7	83.3	100	-	2	8	13	15
13	-	13.3	63.3	83.3		-	4	19	13	
14	-	23.3	80.0	100		-	7	24	15	
17	-	33.3	100			-	10	30		
19	-	46.7				-	14			
21	-	60.0				-	18			
24	-	80.0				-	24			
27	-	83.3				-	25			
32	-	93.3				-	28			
33	-	100				-	30			

Table E.8. Cummulative Mortalities of Macoma Exposed to Et_3PbCl Concentrations Between 0.00 and 1.56 mg l^{-1}

Time	% cummulative mortality				cummulative no. of deaths			
(days)	0.00	0.13	0.30	1.56	0.00	0.13	0.30	1.56
7	-	-	-	12.5	-	-	-	2
10	-	6.3	12.5	31.3	-	2	4	10
13	-	6.3	15.6	31.3	-	2	5	10
17	-	25.0	43.8	93.8	-	8	14	30
18	-	25.0	63.0	93.8	-	8	20	30
19	-	25.0	65.5	93.8	-	8	21	30
20	-	65.6	87.5	93.8	-	21	28	30
24	-	75.0	87.5	100	-	24	28	32
27	-	87.5	93.8		-	28	30	
28	-	93.8	93.8		-	30	30	
32	-	100	93.8		-	32	30	
33	-		100		-		32	

Table E.9. Cummulative Mortality of Scrobicularia Exposed to a) 72 µl TEL l^{-1} and b) 36 µl TEL l^{-1}

a)

Time	% cummulative mortality			cummulative no. of deaths		
(days)	0.0	1	2	0.0	1	2
7	-	13.4	6.7	-	2	1
9	-	26.7	20.2	-	4	3
10	6.7	46.7	26.7	1	7	4
11	6.7	60.0	40.0	1	9	6
14	20.0	100.0	93.3	3	15	14
15	26.7		100.0	4		15
16	46.7			7		
17	100.0			15		

b)

Time	% cummulative mortality			cummulative no. of deaths		
(days)	0.0	1	2	0.0	1	2
7	-	12.5	12.5	-	2	2
9	-	12.5	25.0	-	2	4
10	-	18.8	25.0	-	3	4
11	6.3	43.8	31.3	1	7	5
13	18.8	75.0	50.0	3	12	8
15	25.0	81.3	68.8	4	13	11
16	25.0	87.5	68.8	4	14	11
17	31.3	93.8	68.8	5	15	11
18	37.5	93.8	75.0	6	15	12
21	56.3	93.8	87.5	9	15	14
22	63.0	93.8	87.5	10	15	14
24	68.8	93.8	87.5	11	15	14
29	68.8	100	87.5	11	16	14
46	68.8		87.5	11		14

Table E.10. Cummulative Mortality of Macoma Exposed to a) 72 µl TEL l^{-1} and b) 36 µl TEL l^{-1}

a) Time (days)	% cummulative mortality 0.0	1	2	cummulative no. of deaths 0.0	1	2
7	-	5	-	-	1	-
9	-	5	5	-	1	1
13	-	10	25	-	2	5
15	-	15	30	-	3	6
17	-	20	35	-	4	7
18	-	25	35	-	5	7
21	5	35	35	1	7	7
22	10	35	35	2	7	7
23	10	35	40	2	7	8
25	15	40	40	3	8	8
28	15	65	45	3	13	9
29	15	75	45	3	15	9
31	15	75	55	3	15	11
32	15	75	65	3	15	13
35	15	75	70	3	15	14
39	15	80	70	3	16	14
44	20	80	70	4	16	14
49	20	80	70	4	16	14

b) Time (days)	% cummulative mortality 0.0	1	2	cummulative no. of deaths 0.0	1	2
7	-	5	-	-	1	-
8	-	5	5	-	1	1
9	-	10	10	-	2	2
10	-	10	15	-	2	3
11	-	10	20	-	2	4
14	-	10	65	-	2	13
15	-	15	70	-	3	14
16	-	25	80	-	5	16
17	-	25	85	-	5	17
18	-	30	90	-	6	18
36	-	30	90	-	6	18

E.3.5. Accumulation of Alkyllead Compounds by Scrobicularia and Macoma

Analyses for both species were performed using the technique of Birnie and Hodges[57]. Where more than one individual died on a particular day, all of the animals were bulked and homogenised using a stainless steel rotor. A sub-sample was taken from the homogenate for analysis. Usually three replicate samples were taken to reduce any errors encountered in the analytical procedure. All analyses were based on wet weight soft tissue. When animals were deep frozen under storage , the samples were allowed to thaw before homogenisation.

Table E.11. Accumulation of Me_3PbCl by Macoma (mg Pb/kg wet weight)

Time (days)	0.00	0.04	0.12	0.44
16				10.6,8.7,8.4
17				7.6,8.1,8.9
18			4.8,5.8,5.0	
21				5.9,6.3,6.9
23			0.8,1.3,1.4	
35		0.75,0.45,0.42		
38	<0.02,<0.02,<0.02		1.5,1.2,1.8	
56	<0.02,<0.02,<0.02	0.45,0.60,0.45		

Table E.12. Accumulation of Et_3PbCl by Scrobicularia (mg Pb/kg wet wt.)

Time (days)	Exposure Concentrations (mg l^{-1})			
	0.00	0.14	0.24	1.08
10		1.9, 2.2, 2.2		41.0,41.0,44.0
11			10.8,10.1,8.9	20.0,23.0,28.0
13			7.5, 7.0, 8.0	
14		2.2, 1.8, 2.3	9.6, 7.3, 8.4	
23		4.2, 4.5, 4.2		
34	<0.02,<0.02,<0.02			

Table E.13. Accumulation of Et_3PbCl by Macoma (mg Pb/kg wet wt.)

Time (days)	Exposure Concentration (mg l^{-1})			
	0.00	0.13	0.30	1.56
10				27.0,34.0,29.0
16		4.2, 4.4, 4.6	10.7,14.1,11.2	41.0,50.0,46.0
19		5.4, 6.9, 5.9	17.5,18.7,17.9	
24				46.0,49.0,58.0
28	<0.02,<0.02,<0.02	6.4, 7.0, 7.1	16.2,16.2 15.6	

EXPERIMENTAL WORK RELATING TO CHAPTER FOUR

E.4.1. Siphonal Preparations

The apparatus used to obtain siphonal responses to alkyllead compounds included the following:

Isotomic transducer (C.F. Palmer)

Oscillograph (George Washington 400 MD/2)

Organ bath 150 cm^3 capacity, made of perspex

Pulley wheel made of PTFE

Pasteur pipette tip to aerate and mix the seawater

Aquarium air pump (Rena 301)

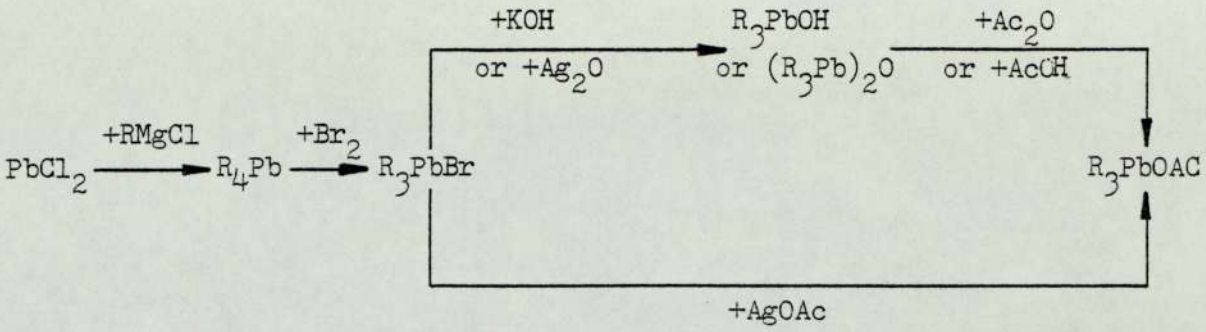
Examples of traces produced from isolated and insitu siphonal preparations exposed to a range of alkyllead compounds are shown in Chapter 4.

E.4.2. Attempted Synthesis of Trihexyllead Chloride

The synthesis of trihexlyllead chloride was attempted in order to further test the influence of alkyl group chain length on toxicity.

There are few examples of the compound in the literature[142], although several studies have been concerned with the synthesis of longer length alkyllead compounds[1,143,144,145,146,147,148]. Several preparative methods are availabe for organolead salts and these include: cleavage of TAL compounds by halogens or acids, cleavage of hexaalkyldilead compounds by oxidising agents, metathesis, cleavage by metal salts and partial organydation.

Cleavage of TAL or hexaalkyldilead (R_6Pb_2) are the most commonly used routes. Kaars Sijesteijn[142] prepared several alkyllead acetates using the following general reaction scheme:



Trihexyllead acetate prepared from this method was reported as crystaline at room temperature, with a melting point in the range 49°C - 57°C.

Our attempts at $(Hexyl)_3PbCl$ were based on the method for the synthesis of tributyllead chloride reported by Willemsens and Van der Kerk[147]. This route involves the cleavage of hexahexyldilead using chlorine gas.

The first stage involved the synthesis of hexahexyldilead via a classical Grignard reaction.

69.5 g ($\frac{1}{4}$ mole) $PbCl_2$ + 68.5 g ($\frac{1}{2}$ mole) hexylbromide + 12.5 g Mg in 250 cm^3 dry diethyl ether.

The $PbCl_2$, Mg and about 50 cm^3 ether were transferred to a 1 litre, 3 necked round bottomed flask and stirred with a mechanical stirrer. The hexylbromide was added slowly until the reaction commenced. Stirring was increased and the remaining ether added. The reaction was allowed to proceed under reflux for 2 hours.

The mixture was chilled to -60°C by adding lumps of dry ice to the flask and placing this in a dry ice/acetone bath. The next stage required Cl_2 to be bubbled through in order to cleave the hexahexyldilead forming the trihexyl product. Cl_2 was synthesised in a side vessel by dropping concentrated hydrochloric acid onto potassium permanganate.

Nitrogen was passed over the mixture to reduce the fire hazard of Cl_2 reacting with the ether.

During the Cl_2 addition, the mixture acquired a dark orange colour but returned to a pale yellow after chlorination had finished. The ether layer was removed after reaching room temperature and was washed with water. On standing overnight at 4°C, the ether layer produced a white precipitate. This precipitate was separated and its melting point determined (180°C - 200°C), which suggested it was an inorganic compound.

On removal of the ether by reduced pressure distillation (Rotorvap) a yellowish oily liquid was obtained. On standing overnight this liquid also produced a whitish crystaline solid of similar appearance and aroma to trialkyllead chlorides. Infra red spectra of the solid suggested that it had little organic character, however, elemental analysis (for C, N and Cl) produced ratios very similar to dihexyllead dichloride.

Actual % elemental content

Sample 1	C - 32%	Sample 2	C - 31.8%
	H - 5.6%		H - 5.7%
	Cl - 15.9%		Cl - 18.8%

(Samples 1 and 2 were prepared separately.)

Expected % elemental content for Trihexyllead Chloride and Dihexyllead Dichloride

Trihexyllead chloride	C - 43%	Dihexyllead dichloride	C - 32%
	H - 7.8%		H - 5.8%
	Cl - 7.1%		Cl - 15.8%

The solid was most probably, therefore, dihexyllead dichloride. Excess chlorination may have caused any trihexyl itself to undergo substitution, the apparently pure dihexyllead resulting. Although several attempts were made to reduce chlorination, the same result occurred.

EXPERIMENTAL WORK RELATING TO CHAPTER FIVE

E.5.1. Culture of Phaeodactylum tricornutum

The strain cultured and used for tests was LB 1052/1, obtained from the Culture Centre for Algae and Protozoa, Cambridge.

The medium used (AE50) was a mixture of two prepared sterile media as follows:

Stock Solutions

Major salts:	NaCl	290g
	$MgSO_4.7H_2O$	80g
	KCl	9.6g
	$CaCl_2.6H_2O$	8.76g
	distilled water	4 litre
Minor salts:	$NaNO_3$	5g
	K_2HPO_4	0.5g
	distilled water	100g
Metals:	$FeCl_3.6H_2O$	0.77g
	$ZnCl_2$	0.0624g
	$MnCl_2.4H_2O$	0.86g
	$CoCl_2.6H_2O$	0.0024g
	$CuCl_2.2H_2O$	0.00064g
	H_3BO_3	6.84g
	Na_2 EDTA	6.0g
	distilled water	4 litre
Tris:	Tris (hydroxy methyl) amino methane	
	$NH_2.C.(CH_2OH)_3$	40g
	distilled water	1 litre

Vitamins:	Thiamine	0.01g
	Nicotinic acid	0.002g
	Calcium pantothenate	0.002g
	p-aminobenzoic acid	0.0002g
	Biotin	0.00002g
	Inositol	0.1g
	Folic acid	0.00004g
	Thymine	0.06g
	B_{12}	0.00004g
	distilled water	100 cm^3
ASP2 Medium:	distilled water	1400 cm^3
	major salts	500 cm^3
	tris	50 cm^3
	metals	40 cm^3
	minor salts	2 cm^3
Erd Schreiber medium:	seawater	11000 cm^3
	soil extract	50 cm^3
	$NaNO_3$	0.2g
	Na_2HPO_4	0.0118g

Preparation of AE50 Medium

An equivolume mixture of ASP2 and Erd Schreiber media was autoclaved at 15 PSI for 20 minutes. pH was adjusted to 7.6 - 7.8 using concentrated HCl prior to autoclaving.

Addition of 0.1% -D glucose and 3 cm^3 sterile vitamin solution completed the preparation.

Stock cultures were maintained in conical flasks under daylight illumination (not windowsill), at room temperature. Aeration was from

aquarium pumps (Rena 301) supplying filtered laboratory air.

E.5.2 ^{14}C Uptake Procedure

Details of the procedure are given in Chapter 5.

After 6 hours incubation under 2000 lux (Thorn warmtone tube) or darkness (tubes wrapped in aluminium foil) the algae were filtered through 0.45 μ Sartorius cellulose nitrate membrane filters held in 25 mm Millipore filter holders. The filters and algae were dissolved in 10 cm^3 Cocktail 'T' (B.D.H.) scintillation cocktail and counted in glass vials using a Beckman 7500 microprocessor controlled LSC. Counts were of disintegration per minute (DPM).

Algal counts prior to incubation were estimated using a Neubauer improved heamacytometer viewed at x 100 under a W. Watson & Sons (London) light microscope.

E.5.3. Effects of Alkyllead Compounds on Photosynthetic Uptake of Phaeodactylum tricornutum

The following data are the results from ^{14}C uptake inhibition studies with a range of alkyllead compounds and other materials. The data are presented in a summarized format excluding some of the calculation steps used to obtain percentage photosynthetic activity as compared to control incubated cultures (100% activity). Counts in brackets at the top of each exposure concentration column are non-photosynthetic ^{14}C uptake (i.e. dark cultures). These are subtracted from the photosynthetic (light) uptake before calculation of percentage activity is made. (*- not included in calculations) Final activity ± 1 S.D.

Et_2PbCl_2

Exposure concentration (mg l^{-1})			% Photosynthetic activity	
4.0	1.0	0.0	4.0	1.0
Count (DPM)				
(71.9)	(98.1)	(77.1)	18.9	56.3
381.4	927.0	1530.4	±3.5	±1.9
287.9	883.9			
371.3	937.0			

Example of calculation procedure:

The non-photosynthetic uptake (e.g. 4.0 mg l^{-1} = 71.9 DPM) is subtracted from the photosynthetic uptake (381.4, 287.9 and 371.3) giving the corrected values (309.5, 216.0 and 299.4). These values are expressed as a percentage of the corrected control ^{14}C uptake (1453.3 DPM) giving percentage activity (21.3, 14.9 and 20.6). The mean of these percentages is taken as the activity of Phaeodactylum exposed to 4.0 mg l^{-1} Et_2PbCl_2 (18.9 ± 3.5%).

Exposure concentration (mg l^{-1})		% Photosynthetic activity
2.0	0.0	2.0
(91.3)	(77.5)	45.1
1084.5	2078.1	±3.3
908.0		
1032.3		
972.1		
975.7		

Me_3PbCl

Exposure concentration ($mg\ l^{-1}$)			% Photosynthetic activity	
2.5	0.2	0.0	2.5	0.2
(253.3)	(329.9)	(415.9)	9.2	79.4
1332.9	8114.1	11603.6	±0.6	±6.3
1184.3	9702.0			
1315.3	8885.4			
* 1519.0	9952.3			
1294.7	9388.6			

Exposure concentration ($mg\ l^{-1}$)			% Photosynthetic activity	
2.0	1.0	0.0	2.0	1.0
(191.4)	(161.6)	(268.5)	10.3	25.6
622.5	1193.1	4383.6	±1.3	±2.9
659.8	1428.3			
673.0	1119.5			
576.6	1177.2			
547.7	1162.6			

Exposure concentration ($mg\ l^{-1}$)			% Photosynthetic activity	
1.5	0.5	0.0	1.5	0.5
(166.6)	(216.9)	(248.9)	8.4	48.3
600.0	2395.4	5097.6	±0.4	±2.7
584.6	2767.3			
568.8	2534.9			
541.1	2600.3			
586.1	2494.3			

Et_3PbCl

Exposure concentration ($mg\ l^{-1}$)			% Photosynthetic activity	
2.0	0.75	0.0	2.0	0.75
(167.4)	(163.6)	(261.2)	0.6	4.6
181.4	358.6	4740.0	±0.6	±0.5
237.1	377.2			
156.2	348.6			
209.7	389.5			
215.9	414.4			

Exposure concentration ($mg\ l^{-1}$)		% Photosynthetic activity
0.5	0.0	0.5
(144.5, 201.8)	(197.9, 204.1)	16.5
860.1	4668.9	±1.0
858.3	4240.8	
856.3	4499.9	
772.7	4594.2	
885.5	4309.4	

Exposure concentration ($mg\ l^{-1}$)			% Photosynthetic activity	
1.0	0.1	0.0	1.0	0.1
(181.4)	(198.1)	(334.7)	5.9	49.6
400.6	2625.2	5677.3	±1.0	±5.6
487.5	3257.9			
372.3	2546.3			
460.6	3044.4			

Exposure concentration (mg l^{-1})			% Photosynthetic activity	
1.5	0.25	0.0	1.5	0.25
(235.7)	(234.9)	(288.0)	0.9	40.1
339.1	* 1179.6	4578.3	±0.7	±1.8
271.0	2005.5			
307.4	2064.9			
272.2	1879.0			
285.0	2006.9			

Bu_3PbOAc

Exposure concentration (mg l^{-1})			% Photosynthetic activity	
1.0	0.5	0.0	1.0	0.5
(196.1)	(155.7)	(315.5)	0.0	±0.2
190.1	132.6	6026.8		
178.7	162.1			
169.1	148.0			
214.0	193.0			
201.3	178.2			

Exposure concentration (mg l^{-1})			% Photosynthetic activity	
0.2	0.1	0.0	0.2	0.1
(96.9)	(111.7)	(139.2)	0.6	3.3
126.8	201.5	2581.5	±0.5	±0.5
* 201.2	209.1			
103.8	186.6			
115.2	181.4			
99.7	189.3			

Exposure concentration (mg l^{-1})			% Photosynthetic activity	
0.05	0.01	0.0	0.05	0.01
(107.7)	(122.1)	(115.9)	5.9	24.0
186.0	602.7	1812.1	±0.9	±6.7
186.9	438.3			
167.9	417.5			
205.9	663.5			

Exposure concentration (mg l^{-1})			% Photosynthetic activity	
0.005	0.001	0.0	0.005	0.001
(123.0)	(171.9)	(141.4)	52.3	95.8
3076.5	5552.7	5617.7	±2.7	±4.9
3149.8	5384.4			
2834.1	5638.9			
2888.8	5556.4			
	4954.7			

TEL

Exposure concentration (µl TEL l^{-1})			% Photosynthetic activity	
125.0	62.5	0.0	125.0	62.5
(86.4)	(167.4)	(85.7)	29.2	36.7
1107.6	1558.2	3812.0	±6.4	±3.9
947.0	1338.6			
1072.2	1434.0			
1572.0	1576.9			
1203.3	1721.1			

Exposure concentration (µl Tel l^{-1})				% Photosynthetic activity	
62.5 (+)	0.0 (+)	62.5 (-)	0.0 (-)	62.5 (+)	62.5 (-)
(142.5)	(180.7)	(144.6)	(152.2)	14.5	35.4
317.9	1213.3	449.8	984.6	$\pm$3.5	$\pm$4.7
292.1		477.4			
304.5		386.7			
253.8		445.6			

(+) or (-) relates to stirred or non-stirred cultures respectively.

Exposure concentration (µl TEL l^{-1})				% Photosynthetic activity	
125.0 (+)	0.0 (+)	125.0 (-)	0.0 (-)	125.0 (+)	125.0 (-)
(133.6)	(160.9)	(136.8)	(143.8)	4.8	20.3
216.9	2242.5	432.0	1964.8	$\pm$1.0	$\pm$3.6
221.4		591.8			
245.8		479.5			
244.8		521.9			

Exposure concentration (µl TEL l^{-1})		% Photosynthetic activity
0.0 (+)	0.0 (-)	$\frac{\text{non-stirred culture}}{\text{stirred culture}} \times \frac{100}{1}$
(180.7)	(152.2)	
(160.9)	(143.8)	85.6 ± 4.4
(135.1)	(155.9)	
1213.3	984.6	
2242.5	1964.8	
2317.5	1015.8	

Et_3PbCl ± stirring

Exposure concentration ($mg\ l^{-1}$)				% Photosynthetic activity	
0.25 (+)	0.0 (+)	0.25 (-)	0.01 (-)	0.25 (+)	0.25 (-)
(155.9)	(135.1)	(109.8)	(118.6)	39.4	39.6
976.4	2317.5	870.8	2058.3	±6.2	±2.0
1225.0		868.6			
969.4		894.1			
892.7		* 714.0			

Et_3PbCl ± Selenium

Exposure concenrtation ($mg\ l^{-1}$)			% Photosynthetic activity	
Et_3PbCl/Se	Et_3PbCl/Se	Control		
0.25/0.0	0.25/10.0	0.0	0.25/0.0	0.25/10.0
(67.1)	(53.4)	71.1)	53.5	38.8
1566.2	1228.9	2953.1	±5.5	±3.3
1877.7	1294.1			
1492.5	1109.2			
1626.0	1172.5			
1488.2	1056.6			

Exposure concentration ($mg\ l^{-1}$)			% Photosynthetic activity	
Et_3PbCl/Se	Et_3PbCl/Se	Control		
0.25/0.0	0.25/1.0	0.0	0.25/0.0	0.25/1.0
(329.3)	(186.5)	(107.7)	43.5	46.0
2408.8	2324.8	4364.1	±2.8	±2.3
2181.3	2091.5			
2221.0	2139.3			
2091.5	2217.5			
2221.0	1847.3			

$\underline{Et_2PbCl_2}$ $\underline{\pm}$ $\underline{Selenium}$

Exposure concentration (mg l^{-1})			% Photosynthetic activity	
Et_2PbCl_2/Se	Et_2PbCl_2/Se	Control		
2.0/0.0	2.0/5.0	0.0	2.0/0.0	2.0/5.0
(91.3)	(61.6)	(77.5)	45.1	47.2
1084.5	1002.7	2078.1	±3.3	±0.9
908.0	992.2			
1032.3	* 790.2			
972.1	1031.5			
975.7	999.9			

Exposure concentration (mg l^{-1})			% Photosynthetic activity	
Et_2PbCl_2/Se	Et_2PbCl_2/Se	Control		
4.0/0.0	4.0/10.0	0.0	4.0/0.0	4.0/10.0
(71.9)	(74.2)	(77.1)	19.0	20.0
381.4	379.6	1530.4	±3.5	±3.2
287.9	313.7			
371.3	402.1			

$\underline{Na_2SeO_3}$

Exposure concentration (mg l^{-1})				% Photosynthetic activity		
1.0	5.0	10.0	0.0	1.0	5.0	10.0
(91.1)	(81.3)	(86.5)	(98.7)	98.7	101.2	85.1
2329.5	2419.9	1998.1	2234.5	±5.3	±7.1	±3.3
2157.1	2132.1	1845.9				
2082.4	2317.5	1853.7				
2228.1	2102.0	1916.5				

$Et_3PbCl \pm$ Sulphide

Exposure concentration ($mg\ l^{-1}$)			% Photosynthetic activity	
Et_3PbCl/S^{2-} 0.25/0.0	Et_3PbCl/S^{2-} 0.25/0.125	Control 0.0	0.25/0.0	0.25/0.125
(100.6)	(81.7)	(93.6)	49.7	57.4
695.3	732.2	1291.3	$\pm$ 4.7	$\pm$ 5.9
702.5	876.4			
633.0	735.9			
740.7	807.7			
707.7	696.6			

Exposure concentration ($mg\ l^{-1}$)				% Photosynthetic activity		
Et_3PbCl/S^{2-} 0.25/0.0	Et_3PbCl/S^{2-} 0.25/0.125	Et_3PbCl/S^{2-} 0.25/1.0	Control 0.0	0.25/0.0	0.25/0.125	0.25/1.0
(145.2)	(111.5)	(91.6)	(159.2)	41.8	41.4	50.2
684.0	586.4	703.8	1373.9	$\pm$ 7.9	$\pm$ 5.9	$\pm$ 6.7
644.7	634.8	743.2				
629.1	*1283.8	655.3				

Exposure concentration ($mg\ l^{-1}$)				% Photosynthetic activity		
Et_3PbCl/S^{2-} 0.25/0.0	Et_3PbCl/S^{2-} 0.25/10.0	Et_3PbCl/S^{2-} 0.0/10.0	Control 0.0	0.25/0.0	0.25/10.0	0.0/10.0
(115.9)	(114.3)	(112.8)	(118.3)	43.2	27.5	69.1
1065.2	836.2	*636.1	2395.4	$\pm$ 2.8	$\pm$ 4.8	$\pm$ 2.1
1155.4	789.9	1700.1				
1076.9	603.3	1671.8				

Exposure concentration (mg l^{-1})				% Photosynthetic activity		
Et_3PbCl/S^{2-}	Et_3PbCl/S^{2-}	Et_3PbCl/S^{2-}	Control			
0.25/0.0	0.25/1.0	0.0/1.0	0.0	0.25/0.0	0.25/1.0	0.0/1.0
(107.4)	(111.2)	(124.3)	(85.6)	44.6	55.7	101.5
311.9	422.6	666.7	605.4	±3.9	±8.8	±5.4
354.7	379.5	610.6				
329.5	452.2	657.2				
360.8	347.9	673.9				

E.5.4. Statistical Significance of Alkyllead Depression of Photosynthetic Activity of Phaeodactylum

Where there were obvious differences between exposed and control cultures, or between different exposure concentrations, statistical significance testing of these differences was not necessary. In other cases where two sets of data appeared similar or not obviously different, the Student's -t test was applied. The following table gives calculated t values, and the level of significance if any, for the various exposure/control/double exposure combinations.

Table E.1

Exposure concentration ($mg\ l^{-1}$)	t value	degrees of freedom	S	level of significance
Et_3PbCl/Se				
0.25/0.0	9.9	8	2.098	S
0.25/10.0				P < 0.001
0.25/0.0	2.09	7	1.58	NS
0.25/1.0				0.10>P>0.05
Et_2PbCl_2/Se				
2.0/5.0	2.05	8	1.45	NS
2.0/0.0				0.10>P>0.05
4.0/0.0	0.86	4	1.8	NS
4.0/10.0				0.50>P>0.20
0.0/1.0	2.5	6	2.45	NS
0.0/5.0				0.05>P>0.02
0.0/1.0	16.6	6	2.02	S
0.0/10.0				P <0.001
Et_3PbCl/S^{2-}				
0.25/0.0	5.03	8	2.16	S
0.25/0.125				0.002>P<0.001

(S = significant, NS = not significant)

EXPERIMENTAL WORK RELATING TO CHAPTER SIX

E.6.1. Medium for Poterioochromonas malhamensis

All Poterioochromonas cultures were maintained in a modified CHU 10 medium (EVT) recommended by the Culture Centre for Algae and Protozoa. The preparation of EVT medium was as follows:

$Ca(NO_3)_2.4H_2O$	20.0 mg l^{-1}
KH_2PO_4	6.2 mg l^{-1}
$MgSO_4.7H_2O$	25.0 mg l^{-1}
Na_2CO_3	20.0 mg l^{-1}
Na_2SiO_3	25.0 mg l^{-1}
N.HCl	0.25 cm^3
$EDTA.Na_2$	2.0 mg l^{-1} } added as complex EDTA.Fe
$FeCl_3$	1.0 mg l^{-1} }
H_3BO_3	2.48 mg l^{-1}
$MnCl_2.4H_2O$	1.39 mg l^{-1}
$(NH_4)_6MO_7O_{24}.4H_2O$	1.00 mg l^{-1}
Vitamin B_{12}	10.0 µg l^{-1}
Vitamin B_1	1.0 µg l^{-1}
Biotin	1.0 µg l^{-1}

The inorganic salts were added to distilled water at the above weights per litre. When all of the material had dissolved it was autoclaved at 15 PSI for 20 minutes. After cooling, the separately made vitamin solution was added. Finally α-D(+) glucose was added to give an overall 0.1% solution.

E.6.2. Apparatus

Algal counts were made using a modified Neubauer haemacytometer ($\frac{1}{400}$ mm^2). The microscope employed was a Watson and Sons Ltd. light microscope. Magnification was X100.

The small samples of algal suspension for counting were taken with an automatic micro-pipette (Finnpipette) with disposable tips.

E.6.3. Cell Counts

Cell counts are given below for all alkyllead toxicity tests. The mean of each culture for each day of incubation is listed, rather than every individual count in order to condense the data. The standard deviation for each mean is also given. All counts are millions of cells per $cm^3 \pm 1$ S.D.

E.6.3.1. Exposure to Et_3PbCl

0.1 mg l^{-1} Et_3PbCl

Initial titer = 0.19 ± 0.02

Day 1

	Et_3PbCl		Control	
Light	0.22 ± 0.08 0.19 ± 0.06 0.21 ± 0.06	0.21 ± 0.06	0.31 ± 0.08 0.30 ± 0.04 0.29 ± 0.08	0.30 ± 0.07
Dark	0.21 ± 0.05 0.19 ± 0.04 0.19 ± 0.04	0.24 ± 0.05	0.21 ± 0.05 0.33 ± 0.08 0.27 ± 0.06	0.27 ± 0.06

Day 2

	Et_3PbCl		Control	
Light	0.21 ± 0.03 0.23 ± 0.06 0.22 ± 0.06	0.22 ± 0.05	0.54 ± 0.08 0.52 ± 0.07 0.51 ± 0.08	0.52 ± 0.08
Dark	0.26 ± 0.03 0.21 ± 0.06 0.23 ± 0.07	0.23 ± 0.05	0.37 ± 0.07 0.58 ± 0.06 0.47 ± 0.06	0.47 ± 0.06

Day 3

	Et_3PbCl		Control	
Light	0.22 ± 0.03 0.22 ± 0.05 0.23 ± 0.05	0.22 ± 0.05	0.60 ± 0.07 0.54 ± 0.07 0.52 ± 0.07	0.55 ± 0.07
Dark	0.23 ± 0.04 0.21 ± 0.05 0.24 ± 0.04	0.23 ± 0.04	0.64 ± 0.08 0.53 ± 0.07 0.71 ± 0.09	0.63 ± 0.08

Day 7

	Et_3PbCl		Control	
Light	0.23 ± 0.07 0.29 ± 0.03 0.25 ± 0.06	0.25 ± 0.05	0.62 ± 0.08 0.69 ± 0.07 0.62 ± 0.08	0.64 ± 0.08
Dark	0.24 ± 0.05 0.18 ± 0.05 0.30 ± 0.03	0.24 ± 0.05	0.58 ± 0.06 0.61 ± 0.04 0.82 ± 0.07	0.67 ± 0.06

$0.1\ mg\ l^{-1}$ Et_3PbCl

Initial cell titer = 0.27 ± 0.02

Day 1	Et_3PbCl		Control	
Light	0.27 ± 0.04 0.36 ± 0.07 0.41 ± 0.08	0.35 ± 0.07	0.37 ± 0.09 0.46 ± 0.09 0.48 ± 0.13	0.44 ± 0.10
Dark	0.35 ± 0.05 0.35 ± 0.06 0.41 ± 0.09	0.37 ± 0.07	0.41 ± 0.05 0.41 ± 0.07 0.49 ± 0.15	0.44 ± 0.09

Day 2	Et_3PbCl		Control	
Light	0.39 ± 0.03 0.38 ± 0.08 0.38 ± 0.10	0.38 ± 0.07	0.45 ± 0.09 0.41 ± 0.06 0.42 ± 0.05	0.43 ± 0.07
Dark	0.40 ± 0.07 0.37 ± 0.07 0.44 ± 0.07	0.40 ± 0.07	0.42 ± 0.04 0.34 ± 0.04 0.42 ± 0.04	0.39 ± 0.04

Day 3	Et_3PbCl		Control	
Light	0.35 ± 0.06 0.37 ± 0.06 0.39 ± 0.04	0.37 ± 0.05	0.39 ± 0.05 0.44 ± 0.09 0.41 ± 0.11	0.36 ± 0.05
Dark	0.36 ± 0.04 0.37 ± 0.05 0.34 ± 0.07	0.36 ± 0.05	* 0.47 ± 0.08 0.28 ± 0.05 0.43 ± 0.04	0.45 ± 0.06

(* not included in the overall mean calculation)

Day 7	Et_3PbCl		Control	
Light	0.34 ± 0.06 0.35 ± 0.09 0.48 ± 0.08	0.39 ± 0.08	0.78 ± 0.06 0.62 ± 0.10 0.45 ± 0.09	0.62 ± 0.09
Dark	0.37 ± 0.03 0.28 ± 0.03 0.37 ± 0.09	0.34 ± 0.05	0.56 ± 0.09 0.34 ± 0.05 0.39 ± 0.07	0.43 ± 0.07

6.0 mg l^{-1} Et_3PbCl

Initial cell titer = 0.15 ± 0.03

Day 1

	Et_3PbCl		Control	
Light	0.01 ± 0.01		0.17 ± 0.03	
	0.01 ± 0.01	0.01 ± 0.01	0.21 ± 0.05	0.20 ± 0.04
	0.01 ± 0.01		0.21 ± 0.04	
Dark	0.01 ± 0.01		0.21 ± 0.06	
	-	0.01 ± 0.01	0.18 ± 0.05	0.18 ± 0.05
	0.01 ± 0.01		0.16 ± 0.04	

Day 6

	Et_3PbCl		Control	
Light	-		0.97 ± 0.15	
	-	0.0	0.61 ± 0.08	0.95 ± 0.29
	-		1.26 ± 0.12	
Dark	-		1.06 ± 0.18	
	-	0.0	0.34 ± 0.08	0.97 ± 0.54
	-		1.54 ± 0.26	

E.6.3.2. Exposure to Me_3PbCl

0.1 mg l^{-1} Me_3PbCl

Initial titer = 0.24 ± 0.06

Day 1

	Me_3PbCl		Control	
Light	0.32 ± 0.07		0.29 ± 0.10	
	0.36 ± 0.09	0.33 ± 0.08	0.37 ± 0.07	0.34 ± 0.08
	0.30 ± 0.09		0.36 ± 0.08	
Dark	0.25 ± 0.05		0.32 ± 0.07	
	0.33 ± 0.06	0.29 ± 0.05	0.35 ± 0.06	0.33 ± 0.06
	0.30 ± 0.05		0.32 ± 0.04	

Day 2

	Me_3PbCl		Control	
Light	0.49 ± 0.08 0.47 ± 0.10 0.49 ± 0.08	0.48 ± 0.09	0.63 ± 0.05 0.55 ± 0.08 0.56 ± 0.10	0.58 ± 0.08
Dark	0.37 ± 0.04 0.41 ± 0.06 0.46 ± 0.07	0.41 ± 0.06	0.51 ± 0.08 0.50 ± 0.08 0.52 ± 0.09	0.51 ± 0.08

Day 3

	Me_3PbCl		Control	
Light	0.49 ± 0.04 0.45 ± 0.08 0.56 ± 0.06	0.50 ± 0.06	0.71 ± 0.11 0.61 ± 0.08 0.69 ± 0.07	0.67 ± 0.09
Dark	0.43 ± 0.02 0.49 ± 0.10 0.57 ± 0.10	0.49 ± 0.07	0.54 ± 0.09 0.65 ± 0.08 0.60 ± 0.09	0.60 ± 0.09

E.6.3.3. Exposure to TEL

62.5 µl TEL l^{-1}

Initial titer = 0.22 ± 0.07

Day 1

	TEL		Control	
Light	0.25 ± 0.05 0.26 ± 0.04 0.23 ± 0.04	0.25 ± 0.04	0.29 ± 0.05 0.23 ± 0.04 0.26 ± 0.02	0.26 ± 0.04
Dark	0.23 ± 0.05 0.26 ± 0.03 0.27 ± 0.04	0.25 ± 0.04	0.26 ± 0.03 0.29 ± 0.05 0.23 ± 0.05	0.26 ± 0.05

Day 2

	TEL		Control	
Light	0.37 ± 0.08 0.34 ± 0.06 0.42 ± 0.05	0.38 ± 0.06	0.59 ± 0.06 0.62 ± 0.07 0.63 ± 0.03	0.61 ± 0.06
Dark	0.53 ± 0.05 0.54 ± 0.09 0.65 ± 0.07	0.58 ± 0.07	0.65 ± 0.10 0.67 ± 0.08 0.67 ± 0.09	0.66 ± 0.09

Day 3

	TEL		Control	
Light	0.37 ± 0.05 0.35 ± 0.07 0.36 ± 0.07	0.36 ± 0.07	0.70 ± 0.08 0.68 ± 0.06 0.67 ± 0.07	0.68 ± 0.07
Dark	0.58 ± 0.05 0.48 ± 0.06 0.68 ± 0.08	0.58 ± 0.06	0.68 ± 0.09 0.70 ± 0.09 0.66 ± 0.09	0.68 ± 0.09

Day 7

	TEL		Control	
Light	0.22 ± 0.06 0.07 ± 0.02 0.09 ± 0.03	0.13 ± 0.04	0.77 ± 0.07 0.75 ± 0.08 0.70 ± 0.09	0.74 ± 0.08
Dark	0.52 ± 0.04 0.35 ± 0.02 0.64 ± 0.06	0.50 ± 0.04	0.70 ± 0.09 0.73 ± 0.07 0.73 ± 0.10	0.72 ± 0.09

6.25 µl TEL l^{-1}

Initial titer = 0.23 ± 0.06

Day 1

	TEL		Control	
Light	0.28 ± 0.04 0.30 ± 0.05 0.25 ± 0.06	0.28 ± 0.05	0.33 ± 0.07 0.36 ± 0.04 0.36 ± 0.06	0.35 ± 0.06
Dark	0.28 ± 0.07 0.40 ± 0.06 0.35 ± 0.04	0.34 ± 0.06	0.31 ± 0.03 0.29 ± 0.04 0.31 ± 0.07	0.30 ± 0.05

Day 2

	TEL		Control	
Light	0.32 ± 0.04 0.28 ± 0.07 0.33 ± 0.05	0.32 ± 0.05	0.54 ± 0.05 0.51 ± 0.08 0.64 ± 0.07	0.56 ± 0.07
Dark	0.38 ± 0.06 0.61 ± 0.08 0.65 ± 0.06	0.55 ± 0.07	0.49 ± 0.08 0.58 ± 0.08 0.64 ± 0.06	0.57 ± 0.08

Day 3

	TEL		Control	
Light	0.23 ± 0.04 0.27 ± 0.05 0.27 ± 0.06	0.26 ± 0.05	0.47 ± 0.08 0.65 ± 0.07 0.63 ± 0.08	0.58 ± 0.08
Dark	0.50 ± 0.07 0.58 ± 0.07 0.67 ± 0.05	0.59 ± 0.07	0.53 ± 0.06 0.63 ± 0.08 0.71 ± 0.08	0.62 ± 0.08

Day 7

	TEL		Control	
Light	-		0.75 ± 0.08	
	0.10 ± 0.03	0.05 ± 0.02	1.05 ± 0.14	0.91 ± 0.13
	0.04 ± 0.01		0.93 ± 0.17	
Dark	0.44 ± 0.04		0.73 ± 0.16	
	0.53 ± 0.07	0.54 ± 0.06	0.86 ± 0.07	0.79 ± 0.11
	0.64 ± 0.08		0.78 ± 0.09	

62.5 µl TEL l^{-1} ± 1.0 mg l^{-1} Fe^{3+} (All dark)

Initial titer = 0.23 ± 0.05

Day 1

	TEL		Control	
$+Fe^{3+}$	0.44 ± 0.06		0.37 ± 0.08	
	0.36 ± 0.04	0.39 ± .06	0.38 ± 0.07	0.40 ± 0.08
	0.38 ± 0.07		0.43 ± 0.09	
$-Fe^{3+}$	0.29 ± 0.05		0.49 ± 0.08	
	0.35 ± 0.08	0.35 ± 0.07	0.44 ± 0.06	0.45 ± 0.07
	0.43 ± 0.08		0.42 ± 0.06	

Day 2

	TEL		Control	
$+Fe^{3+}$	0.47 ± 0.08		0.47 ± 0.09	
	0.48 ± 0.08	0.49 ± 0.08	0.49 ± 0.08	0.49 ± 0.08
	0.53 ± 0.07		0.50 ± 0.08	
$-Fe^{3+}$	0.35 ± 0.05		0.46 ± 0.06	
	0.42 ± 0.05	0.44 ± 0.06	0.47 ± 0.07	0.48 ± 0.07
	0.55 ± 0.08		0.50 ± 0.07	

Day 7

	TEL		Control	
$+Fe^{3+}$	0.74 ± 0.09 0.75 ± 0.09 0.58 ± 0.07	0.69 ± 0.09	1.07 ± 0.12 0.88 ± 0.10 0.84 ± 0.09	0.93 ± 0.10
$-Fe^{3+}$	0.43 ± 0.05 0.37 ± 0.05 0.53 ± 0.09	0.44 ± 0.06	0.48 ± 0.07 0.64 ± 0.09 0.58 ± 0.08	0.57 ± 0.08

E.6.3.4. Exposure to TML

500 µl TML l^{-1}

Initial titer = 0.18 ± 0.03

Day 1

	TML		Control	
Light	0.23 ± 0.07 * 0.08 ± 0.03 0.19 ± 0.04 0.19 ± 0.03 * 0.08 ± 0.02	0.20 ± 0.05	0.37 ± 0.03 0.39 ± 0.03	0.38 ± 0.03
Dark	0.22 ± 0.05 0.18 ± 0.03 * 0.07 ± 0.03 0.25 ± 0.12	0.22 ± 0.07	0.37 ± 0.07 0.32 ± 0.09	0.34 ± 0.08

(* not included in the mean calculation)

Day 2

	TML		Control	
Light	0.27 ± 0.09 0.25 ± 0.08 * 0.12 ± 0.02 0.22 ± 0.05 0.17 ± 0.02	0.25 ± 0.70	0.58 ± 0.09 0.47 ± 0.06	0.52 ± 0.07
Dark	0.43 ± 0.07 0.33 ± 0.07 0.30 ± 0.10 0.44 ± 0.05	0.38 ± 0.07	0.65 ± 0.12 0.59 ± 0.10	0.62 ± 0.11

Day 3

	TML		Control	
Light	0.41 ± 0.08 0.23 ± 0.03 * 0.13 ± 0.05 0.41 ± 0.04 * 0.13 ± 0.03	0.35 ± 0.05	0.81 ± 0.17 0.73 ± 0.14	0.76 ± 0.16
Dark	0.51 ± 0.09 0.49 ± 0.07 0.39 ± 0.13 0.52 ± 0.12	0.48 ± 0.10	0.78 ± 0.15 0.72 ± 0.15	0.75 ± 0.15

62.5 µl TML l^{-1}

Initial titer = 0.22 ± 0.04

Day 1

	TML		Control	
Light	0.34 ± 0.08 0.37 ± 0.07 0.36 ± 0.05 0.36 ± 0.07 0.42 ± 0.07	0.37 ± 0.07	0.35 ± 0.09 0.30 ± 0.07	0.32 ± 0.08
Dark	0.36 ± 0.07 0.28 ± 0.08 0.33 ± 0.09 0.41 ± 0.11	0.34 ± 0.09	0.37 ± 0.12 0.29 ± 0.12	0.33 ± 0.12

Day 2

	TML		Control	
Light	0.45 ± 0.10 0.42 ± 0.12 0.43 ± 0.09 0.39 ± 0.03 0.38 ± 0.06	0.42 ± 0.08	0.39 ± 0.12 0.36 ± 0.11	0.37 ± 0.11
Dark	0.38 ± 0.04 0.36 ± 0.07 0.37 ± 0.07 0.33 ± 0.04	0.36 ± 0.06	0.37 ± 0.10 0.37 ± 0.12	0.37 ± 0.11

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