INTERACTIONS BETWEEN SELECTED METAL COMPOUNDS AND MARINE HETEROTROPHIC BACTERIA

By

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GENERAL ABSTRACT

A method was developed to test the toxicity of several metal compounds to selected bacteria. The technique was based on the agar diffusion method. Reliability and reproducibility were enhanced by careful standardisation of experimental parameters. By quantification of the concentration of metal compounds in the media using sequential strips of agar it was possible to prepare standard graphs (metal concentration vs. distance from disc). These graphs demonstrated the exponential rate of diffusion of metals. The diffusion rates were metal specific. In the case of lead compounds the diffusion rate was poor. A media was developed which allowed lead chloride to diffuse relatively well.

The susceptibility patterns of forty-three strains of environmental bacteria to twelve metal compounds were determined using the modified agar diffusion method. Statistical analysis of the results showed that Gram positive organisms were more susceptible than Gram negative organisms.

Fifty-nine strains of Escherichia coli were isolated from sediments extracted from twelve sites at four Eastern Cape Rivers. These were examined for metal and antibiotic susceptibility using agar diffusion methods. Statistical analysis was employed to test the hypothesis that metal/antibiotic resistant bacteria are maintained due to selective pressure of metals in the environment. With the exception of cobalt, there was no positive correlation of the data to the hypothesis.

No evidence was found of greater susceptibility to metals between laboratory and sediment strains of E. coli.

The modified agar diffusion method was adapted to test for the synergistic and antagonistic effects of metal compounds. Using industrial and domestic effluents as well as standard metal solutions it was possible to demonstrate the effects of metals in combination upon stock bacterial cultures. This was achieved by placing metal impregnated discs upon the agar surface in such a way that any interactions were observed, after incubation, as distortion in zone sizes and/or shapes.

The results demonstrated that many metal compounds, when in combination with other metals, can produce synergistic effects. A few metals showed antagonism and several remained unaffected. The results also indicated that the method has practical application in testing industrial effluents for relative toxicity. The tube dilution method was also used for testing metal toxicity but discarded due to the anomalous results that were obtained.

Limited experiments were performed to determine if environmental strains of bacteria, isolated from Eastern Cape sediments, were capable of bioaccumulation and/or biotransformation of metal compounds.

Axenic cultures of 12 bacterial strains (6 genera) were established in metal-amended media. After filtration, bacterial mass and metal content determinations showed that lead and

selenium were accumulated by some strains but that arsenic was not.

A subsequent experiment using E. coli and lead chloride demonstrated the ability of this organism to bioaccumulate in excess of seven times its own mass of lead.

Biotransformation of cadmium chloride by E. coli was demonstrated using inoculated metal-amended media. After incubation, solvent extracts of the inorganic and organic phase of the metal were analysed for concentration levels. The results indicated that bacteria are able to biotransform some metal compounds at certain concentrations.

CHAPTER 1

1. GENERAL INTRODUCTION

The aim of this study was to determine the interactions of a wide range of metal compounds with selected heterotrophic sediment bacteria.

Metal compounds are essential to the development and survival of all life forms. Even trace quantities can exert both positive and negative effects on plant and animal life. However, total metal concentrations present in the environment do not necessarily reflect the impact of these compounds on the biota (Sibley & Morgan, 1975). Therefore concurrent with direct metal ion measurements biological systems must be employed to determine the effect of metals on the environment. Bacteria play a major role in geochemical and biological recycling and can alter the form of certain metal compounds. It is essential therefore to study the interactions of heavy metals and bacteria. These interactions will include the effects of the metals upon different bacterial species and the effect of bacteria on different metal compounds.

Heterotrophic bacteria are ubiquitous and exist in marine systems under a wide range of physical and chemical conditions. Metals enter the environment from at least five major sources (Wittman 1981(b)):

1) geological weathering
2) industrial effluents
3) leaching from waste
4) human and animal excrement
5) mining industry

Geological weathering of surface and near surface rocks results in a low level introduction of metals into the environment. Many of these metals are subsequently deposited in marine sediments. Laitinen (1973) stressed the need to differentiate between naturally occurring levels and man-made levels when measuring pollution. The proportions of these two levels vary according to the individual metal (Fleischer, 1973). Similarily, variations will occur in the average concentration of individual metals found in a study area containing several distinct sources of input (Young et al. 1973).

Our knowledge of the total spectrum of interactions between metal ions, metal compounds and the biota is incomplete. However, there are many documented cases of the disastrous consequences to life that have occurred due to the input of metals into the environment as a result of man's activities (Wittman, 1981(a)). It has therefore been necessary to adopt standards for water quality criteria. Reference can be made to these criteria for determining toxic levels of selected metals to marine and animal life (Kempster et al. 1980). Such standards are based on available research data which themselves are directly reliant upon analytical methodology. To

lead to a greater understanding of metals in the biological context the techniques in use must be able to detect very low concentrations of metal ions and metal compounds accurately. Atomic absorption spectroscopy is such a technique and its application from the first published references in 1958/1959 to its present day development in speciation detection is discussed by Willis (1978). Improvements in the technique have allowed the quantification of metals to the picogram level (L'vov, 1978; Massmann, 1968; Watling, 1975).

The use of such sensitive techniques has enabled many workers to study metal toxicity in a wide range of biological systems, e.g. human life (Norval, 1978; Wittman, 1981(a)); marine life (Chapman, 1978; Marchetti, 1978); marine larvae (Conner, 1972; Brown & Newell, 1972; Calabrese et al. 1973); phytoplankton and plants (Overnell, 1976; Bartlett, 1974; Jensen et al. 1974, 1976); bacteria (Griffiths et al. 1974; Houba & Remacle, 1980).

Many studies demonstrate the ability of certain biota to detoxify metal compounds by biotransformation. However, the biotransformed metal compounds may have enhanced toxicity to other species. Similarily, many organisms bioaccumulate toxic metal compounds in selected tissues as a means of detoxification. These bioaccumulated metals may be toxic to organisms at higher trophic levels.

Evidence of the ability of bacteria to biotransform metal

compounds is presented by Summers & Silver (1978). They reviewed the microbial transformations of toxic and non-toxic metal cations and divided the transformations into two categories; redox conversion of inorganic metal forms and inter-conversions from inorganic to organic forms. The metals included in the latter category were mercury, lead, cadmium, tin, arsenic, selenium and tellurium. Dunn & Bull (1983) showed that bacteria, isolated from activated sludge, had the ability to bioaccumulate Cu^{2+} ions and postulated the use of bacteria in the recovery of copper from industrial wastes. Aspects of the interactions of metals and bacteria are discussed in a review by Gadd & Griffiths (1978) who stress the role of environmental factors in influencing metal toxicity and discuss the various mechanisms of microbial resistance. In a review by Sterritt & Lester (1980) the factors that influence the form and species of a metal, e.g. pH, chelation, are emphasized. Both reviews discuss the role of plasmids in bacterial resistance to metal toxicity. Mietz & Sjogren (1982) discuss the co-selection for antibiotic resistant bacterial populations, that have plasmid mediated metal resistance, as a result of selective pressure from metal polluted environments.

Mills & Collwell (1977) showed that high levels of metals can cause acute changes in the microbial activity of marine ecosystems. Therefore an understanding of the survival of different species of bacteria in metal-rich ecosystems is essential in order to predict the effects of man-made pollution upon any given environment.

To study the various aspects of metal/bacterial interactions it is necessary to determine the ability of bacteria to tolerate varying concentrations of metal compounds, either as single compounds or as multicomponent mixtures. Also, the bioaccumulation and biotransformation of metal compounds by bacteria and the effects of these mechanisms must be assessed. At the same time the relationships between antibiotic resistance, metal resistance and the presence of plasmids must be fully investigated.

Due to the ubiquitous nature of bacteria and the large number of metal compounds in existence extensive research is required in order to obtain a better understanding of bio-accumulation and biotransformation processes by micro-organisms. Estimations of the levels of metal concentrations to which bacteria are tolerant can be used to determine base level metal compound concentrations for use in such bioaccumulation and biotransformation experiments.

Several methods of determining the resistance of bacteria to metal compounds are available. Tan (1980) used dilutions of sea water and sediments from continuous flow cultures, which had been spiked with lead, to estimate total viable counts. These were measured in agar by direct counting and in broth by the "most probable number" (MPN) technique. With the use of selective media he demonstrated the action of Pb^{2+} on aerobic heterotrophic, anaerobic heterotrophic, aerobic

nitrogen fixing, chitinolytic, proteolytic, cellulolytic, nitrate reducing, sulphate reducing and denitrifying bacteria. Mayfield et al (1980) also used continuous culture techniques to demonstrate the effect of mercury (5 and 10 mg l^{-1}) on mixed cultures of sediment bacteria. The effects of mercury were assessed by optical density reading of the cultures, total viable counts measured by plating on soil extract agar, and biomass measurements. Estuarine water was examined for glucose mineralisation and glucose uptake in the presence of copper by Goulder et al (1979). They found a significant negative correlation between glucose mineralization per mass of bacteria and copper concentration. Bacterial mass was estimated from the results of epifluorescent microscopy counts. Glucose mineralization was measured using the method described by Harrison et al (1971). To examine chemical speciation in metal amended soils Lighthart et al (1983) prepared terrestrial microcosms. After nine days incubation at 20°C these were amended with aqueous solutions of 25 different metal compounds each at four selected concentrations. The effect of these compounds was determined by measurement of respiratory inhibition using acid titration of CO_2 levels in each microcosm. Antibiotic-resistant bacteria isolated from sea water were tested for metal resistance by Simon-Pujol et al (1980). They used pure cultures of Pseudomonas aeruginosa and Escherichia coli from sea water and determined metal resistance by the agar dilution method using Mueller-Hinton agar. Twelve metal compounds, each at one selected concentration, were used for the test.

Many other methods have been used to determine metal resistance of bacterial isolates. There is a great deal of experimental variation, not only in the techniques used, but also in the type and concentration of metals, media and micro-organism. Both absorption by and adsorption onto soil particles may give rise to anomalous results using soil based media and media constituents themselves may bind metal ions or cause their precipitation (Seyfried, 1980; Hallas et al 1982). Therefore, there is a need to standardise tests used to determine bacterial metal susceptibility and for a rationale for interpretation of the results of these tests. Some attempts have been made in this direction by Duxbury (1981). He employed the formula

$$y = ae^{-bx}$$

where a = number bacteria/g soil in unamended media, y = number bacteria/g soil in media containing x (mM) concentration of metal and b is an exponent which is a measure of toxicity. By extrapolation to a value of y=1, concentrations of metal to which a bacterium could be considered tolerant could be determined.

Seyfried (1980) suggested that the agar dilution technique was an accepted procedure for detection of metal resistance among micro-organisms but that the use of complex nutrient agar had certain shortcomings.

As the aim of this study was to examine metal/bacterial

interactions, it was decided to develop a method that would enable the toxicity of a large number of metal compounds to be tested with several bacterial isolates. The agar diffusion method for determining susceptibility of bacteria to antibiotics was modified for this purpose. Using this modified method the resistance patterns of environmental and laboratory stock cultures of bacteria to a range of metal compounds were determined. Synergistic and antagonistic effects of metals to bacteria and bioaccumulation and biotransformation of metal compounds by bacteria were also studied using this method.

CHAPTER 2

2.1 INTRODUCTION

The study of the interactions of heavy metals and bacteria may be divided into two -

(a) The effects of metal compounds on bacteria

(b) The effect of bacteria on metal compounds

Since bacteria are involved in both geochemical and organic nutrient recycling processes an understanding of metal/bacterial interaction is fundamental to assessing the environmental impact of metal-loaded effluents. Any disturbance in the equilibrium of the microbial ecology of an ecosystem can have detrimental effects to that ecosystem.

The possible effects of man's input of metal compounds into the environment may be determined directly by bioassay (Bauer et al 1981; Thompson & Watling (in press); McElroy 1983) or indirectly by enumeration of resistant bacteria in metal contaminated and uncontaminated environments.

Direct bioassay techniques are the more simplistic of the two approaches. Nevertheless in order to assess the environmental impact of effluents it is necessary to measure not only chemical but also biological effects.

More detailed information is often obtained when studies of relationships between different environments and the incidence of metal resistant bacteria are carried out. One

such study by Houba and Remacle (1980) examined three fresh water systems for the ability of resident bacteria to develop in the presence of cadmium. The results showed some relationship between numbers of resistant strains and levels of toxicity. Hallas & Cooney (1981) examined sediment and water samples for tin resistant organisms from both fresh water and estuarine areas of Chesapeake Bay. They concluded that there was not always a relationship between increased levels of metals and increased bacterial resistance.

Kurata et al (1977) demonstrated an increase in nickel-tolerant bacteria in nickel-polluted sediments compared to unpolluted sediments. Similarily a positive correlation between mercury resistant bacteria and sediment-mercury levels was found by Nelson & Colwell (1975).

The co-selection of antibiotic resistant strains of bacteria in metal-polluted environments has been reported by several workers (Devanas et al 1980; Simon-Pujol et al 1980). The role of plasmids in bacterial resistance to both antibiotics and metals has been a topic of much research. Transferable antibiotic resistance in coliform bacteria in urban and remote Xhosa communities was discussed by Burt & Woods (1976). Transferable drug resistance in faecal coliform, coliform and Salmonella strains isolated from canal water and bottom sediments was demonstrated by Goyal et al (1979). The presence of plasmid

mediated metal ion resistance in both Gram negative organisms and Gram positive organisms (Tetaz & Luke 1983; Foster 1983, Silver et al 1981) has been recorded.

The effects of bacteria on metals has also been the subject of much research. The ability to biotransform and/or bioaccumulate metal compounds may be used by some bacteria as a means of detoxification in metal polluted environments. Transformations of metals from inorganic to organic forms as a result of bacteriological action are well documented, (Barkay et al 1979; Olson et al 1981; Yamada & Tonomura 1972).

The bioaccumulation of metals and their transferrence through the food chain to higher trophic levels has been demonstrated by Patrick & Loutit (1976). Metal reclamation by bacteria has been discussed by several workers (Baldry & Dean 1980; Remacle & Houba 1983; Guay & Silver 1977).

A factor limiting to many metal/microbe studies is the time and equipment needed to obtain sufficient data for a large number of bacterial species and metal compounds.

The aim of this study was to develop a simple, rapid and reliable method whereby the bacterial resistance to a large number of metals could be determined. It was postulated that provided minimum inhibitory concentration (MIC) readings could be determined by the method as investigated in this study,

then these levels could act as a base for bioaccumulation and biotransformation experiments. Agar dilution and tube dilution are two methods that have been commonly used to determine bacterial resistance. Broth dilution is used in pharmaceutical research as a means of measuring preservative toxicity (Zeelie & McCarthy 1983) and is also used for antibiotic susceptibility testing (Turck et al 1963). The toxicity of zinc, cadmium and lead to heterotrophic soil bacteria was assessed by Olson & Thornton (1981) who used the agar dilution method. Other alternative methods that have been used are biological oxygen demand (BOD) (Bauer et al 1981; Damyanova 1982); measurements of CO_2 respiration (Lighthart et al 1983) and measurements of substrate utilization (Parkin et al. 1983).

Cherdyntseva (1982) evaluated the toxic effect of zinc on Pseudomonas fluorescens by measurements of total counts, assimilation of carbon dioxide and percentage survival of cells using differential staining techniques.

The toxicities of five elements to bacteria were investigated by Smith et al (1982) who modified the antibiotic susceptibility test of Bauer et al (1965). This study describes further modifications that were made to this agar diffusion method, including the standardisation of experimental parameters and the quantification of the metal concentrations across the inhibition zone. The modified agar diffusion method was applied to determine bacterial sensitivity to zinc,

arsenic, manganese, nickel, chromium, copper, cadmium, mercury, selenium, lead and cobalt using E. coli as test organisms. MIC levels of these elements were determined for E. coli by reference to standard graphs. The graphs were prepared by regression analysis of data obtained from measurement of metal concentrations in agar strips.

Seyfried (1980) discussed the metal binding capacity of certain media constituents and the need for further study of their influence on interactions between metals and bacteria. In laboratory tests, the availability of the metal to the micro-organism is obviously a major factor in determining the MIC. Metal compounds that appeared to be non-reactive either in agar or broth, possibly due to binding or chelation of the elements, were tested in alternative media.

The modified agar diffusion method was used to determine the effects of each of twelve metal compounds on 43 isolates of heterotrophic sediment bacteria. The Mann-Whitney test statistic was applied to zone size measurements obtained for each metal. The differences between susceptibility of Gram positive and Gram negative organisms were thereby determined.

2.2 MATERIALS AND METHODS

2.2.1 Stock cultures of bacteria

Escherichia coli NCTC 10418 was maintained on tryptone soya agar at room temperature.

2.2.2 Media

All media used are listed in the Appendix.

2.2.3 Metal solutions

Stock solutions of metal compounds were prepared in distilled water using analar grade reagents. The concentrations for each metal were at 1000 µg/ml for the chlorides of zinc, manganese, nickel, lead, cobalt, cadmium and copper and for selenium as selenium dioxide and chromium as chromium trioxide respectively; at 2000 µg/ml for arsenic as sodium arsenate; at 500 µg/ml and 100 µg/ml for mercury as methyl-mercuric chloride and mercuric chloride respectively. The stock solutions were sterilised by filtration through 0,45 µm Millipore filters.

2.2.4 Paper discs

Whatman AA discs (diameter 13 mm) were sterilised in a hot oven at 160°C for one hour.

2.2.5 Inoculum preparation

E. coli stock culture was grown on MacConkey agar for 18 h to determine purity. A few representative colonies were subcultured into tryptone water and

incubated at 37°C for 18 h. A standard inoculum was prepared by transferring 0,1 ml of the E. coli subculture to a fresh 5 ml volume of tryptone water immediately prior to the test. For Gram positive organisms the volume of subculture added was increased from 0,1 ml to 0,5 ml.

2.2.6 Isolation and identification procedure for bacteria in sediments

Sediment was extracted from six sites in Swartkops River (Fig. 2.1) to a depth of 100 mm using pre-sterilised PVC corers (360 mm x 20 mm). The sediment was transported in sterile bottles to the laboratory where approximately 1 g of sediment was added to each of two Robertsons ACM media. Incubation was carried out at 20°C for 48 h and 37°C for 18 h. Subcultures were made on to nutrient agar and MacConkey agar. Pure colonies of bacterial growth were subcultured onto nutrient agar slopes prior to testing for metal susceptibility by agar diffusion. Identification of the cultures was achieved by Gram stain reaction and subsequent use of the API system (API 20E) for Gram negative bacteria. Gram positive bacteria were grouped according to spore morphology.

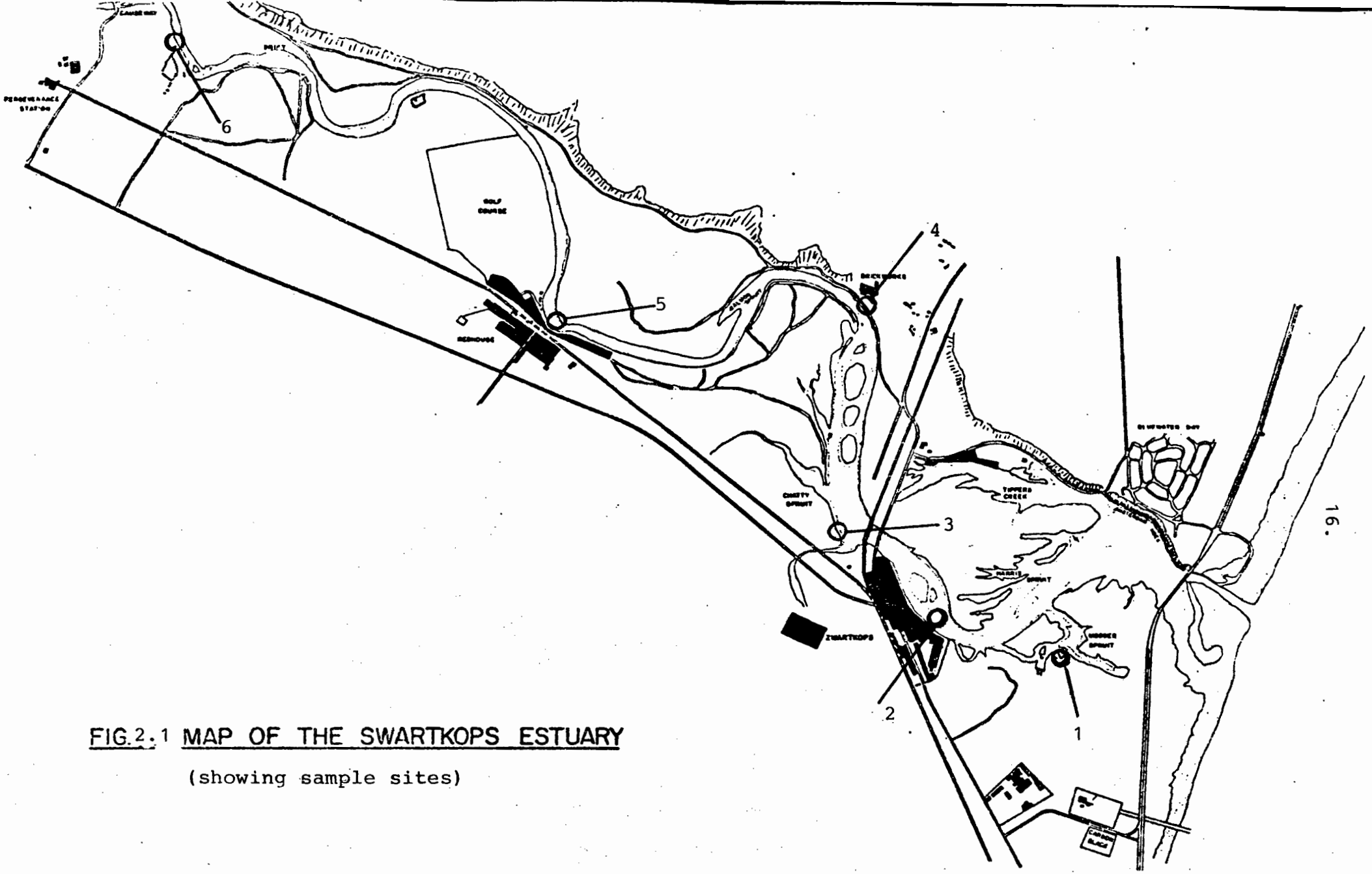


FIG.2.1 MAP OF THE SWARTKOPS ESTUARY

(showing sample sites)

2.2.7 Metal diffusion gradients

In order to prepare metal diffusion gradients, nutrient agar was melted and cooled to 50°C and 10 ml volumes were transferred aseptically to each petri dish. Metal impregnated discs were prepared in duplicate by dropping 0,1 ml of the stock metal solution onto the disc (stock conc./ 10 (µg) per disc). The discs were placed on the agar surface and the plates incubated at 37°C for 18 h.

After incubation, twenty strips of agar (15 mm x 2 mm) were removed sequentially from the edge of the disc to the edge of the plate. Each strip was weighed, digested with 2 ml concentrated nitric acid in a glass vial and evaporated to dryness. A 2 ml aliquot of 10% v/v nitric acid was added to dissolve the sample residue. The metal concentrations in these solutions were determined by atomic absorption spectrometry. The results were calculated as µg/g metal in agar and the diffusion gradient plotted in terms of metal concentration and distance from the edge of the disc.

2.2.8 Bacterial susceptibility to metals

Agar diffusion was used to test for bacterial sensitivity to metals. Nutrient agar was

melted and cooled to 50°C and 5 ml standard inoculum per 100 ml agar was added. Even distribution of the inoculum was achieved by gentle rotation and 10 ml volumes added to each petri-dish. Metal-impregnated discs, prepared in duplicate as for the diffusion gradients, were placed on the agar surface within 30 min of the preparation of the seeded agar plates and the plates incubated at 37°C for 18 h.

After incubation the zones of inhibition of bacterial growth, as visible to the naked eye, were measured with a micrometer and recorded in mm zone size. The zones were measured from the outer edge of the disc to the inner edge of the normal growth zone and included areas of diffuse growth when these were present. Agar strips were then removed from the plate and metal concentrations determined as described previously.

2.2.9 Analysis of sediments for metal content

Approximately 2 g of air-dried sediment were accurately weighed and transferred to a 250 ml Erlenmeyer flask. Ten millilitres nitric acid were added and the mixture heated at 150°C for 2 h under partial reflux. The reflux head was then removed and the mixture heated to dryness.

When dry 10 ml of a 4:1 mixture of nitric: perchloric acid were added and the mixture evaporated to dryness. The sample was then removed from the hotplate and allowed to cool. When cool 10 ml of 10% v/v nitric acid were added to dissolve the soluble constituents in the dried residue and the mixture transferred to a stoppered vial to await analysis (Table 2.1).

2.3 RESULTS

2.3.1 Standardisation of experimental parameters

Preliminary studies showed that several factors influenced the reproducibility of zone sizes. In an international interlaboratory study on single disc diffusion for antibiotic testing (Ericsson & Sherris 1971) it was concluded that non-standardisation of the inoculum concentration was the biggest single factor in the variability of the results. Tests in this laboratory, using metals instead of antibiotics, have shown that the standard inoculum detailed here yields reproducible results.
A just confluent growth was achieved after incubation for both Gram negative and Gram positive organisms.

The nutrient agar media was selected for this method because it yielded clear, well-defined zones

TABLE 2.1

Metal Concentrations in Sediment from Swartkops River

(µg/g)

Site	Cu	Pb	Zn	Mn	Cd	Fe	Cr	Co	Ni	Hg
1	0,9	1,6	3,2	13	< 0,1	941	2,8	0,4	1,9	0,005
2	4,5	0,2	20,1	167	< 0,1	6515	12,1	2,0	4,8	0,050
3	10,3	1,1	26,7	198	< 0,1	8103	14,7	2,0	4,3	0,035
4	8,1	13,7	34,4	26	< 0,1	5544	7,3	1,3	3,8	0,013
5	8,9	73,4	34,6	41	< 0,1	4522	9,6	0,7	2,7	0,015
6	3,4	9,3	21,7	45	< 0,1	4028	13,5	1,2	4,5	0,021

and allowed most of the metals to diffuse evenly, as shown by the diffusion gradients (Figs. 2.2-2.14). The volume of agar used, and hence depth, was a critical factor in achieving reproducible results. Diffusion with depth was also investigated by placing a metal impregnated disc on 30 mm deep agar and cutting serial sections beneath the disc. The relative decrease in metal concentrations with increasing distance from the disc at the surface was the same as for the horizontal diffusion gradient (Fig. 2.15).

Stainless steel cylinders, porcelain beads and wells cut into the agar surface were tested as alternatives to the paper discs. All these methods gave smaller zones of inhibition of bacterial growth and less reproducible results.

Metal concentrations in individual discs varied by up to 200% when a known volume of standard metal solution was added to several discs together. The addition of the metal solution to each disc, using an Eppendorf pipette immediately prior to placing the disc on the seeded agar surface, ensured that accurate metal concentrations were achieved for each test.

Another factor which influenced zone size was the time lapse between seeding the agar with the inoculum

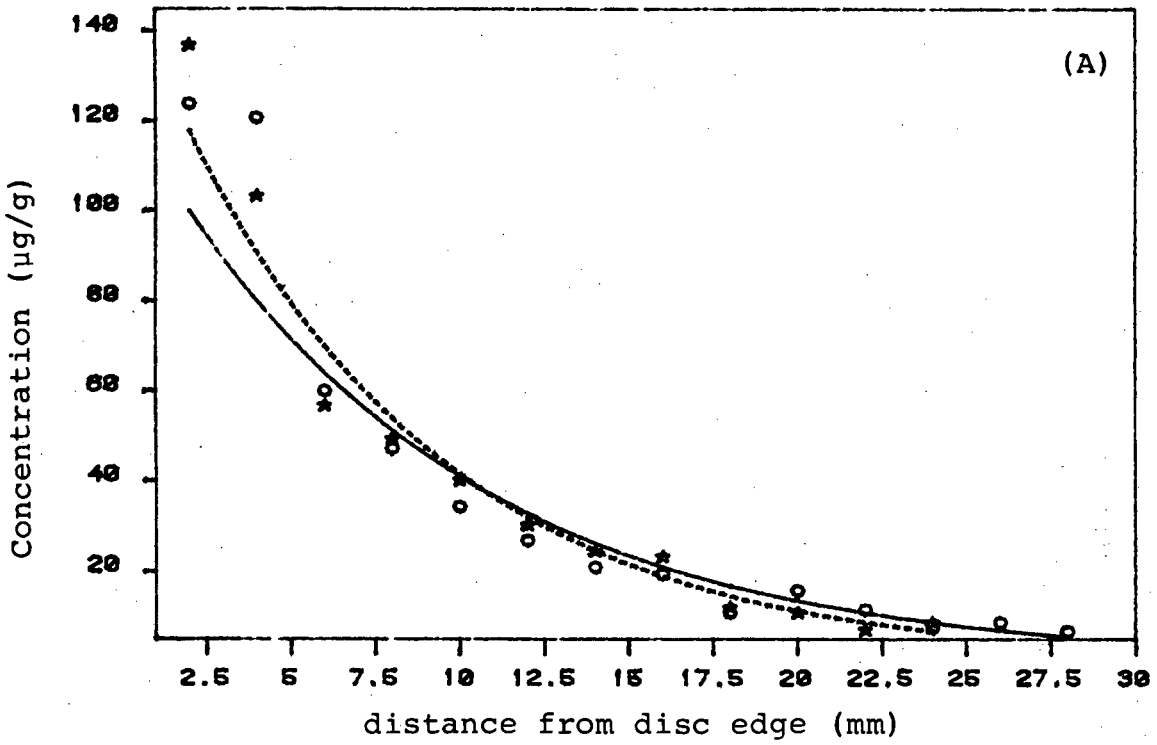
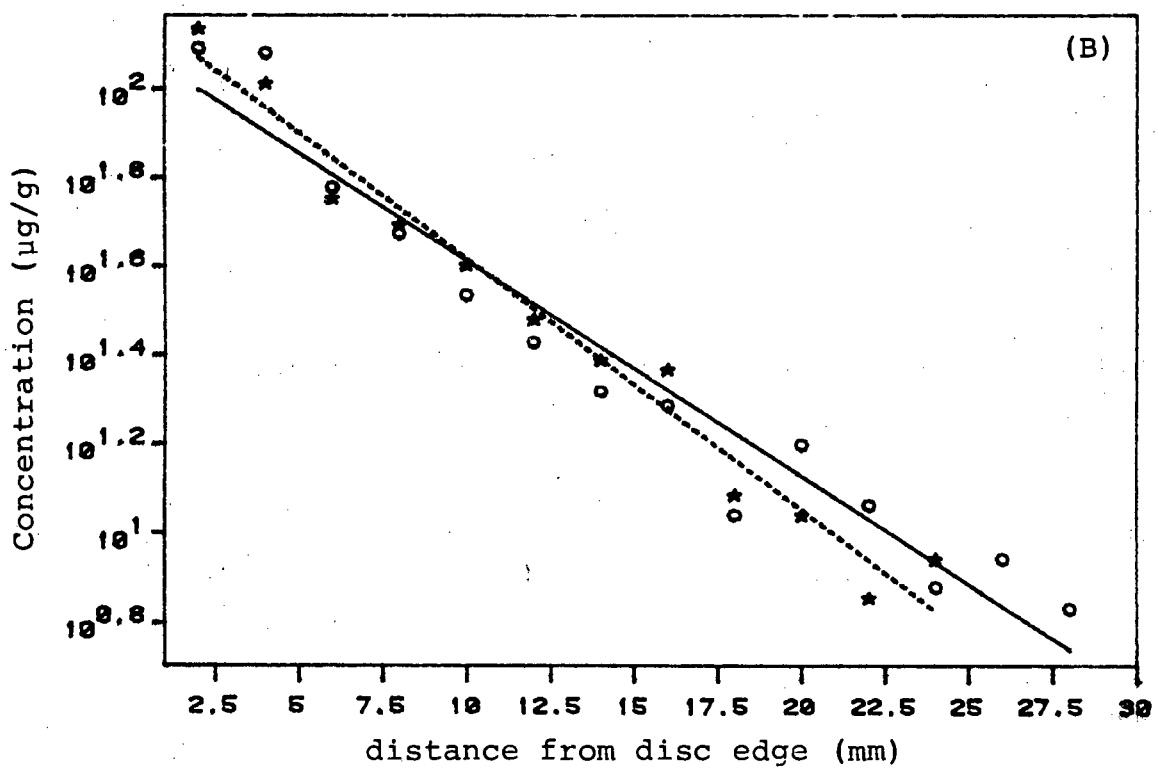


Fig. 2.2 Diffusion gradients for copper chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



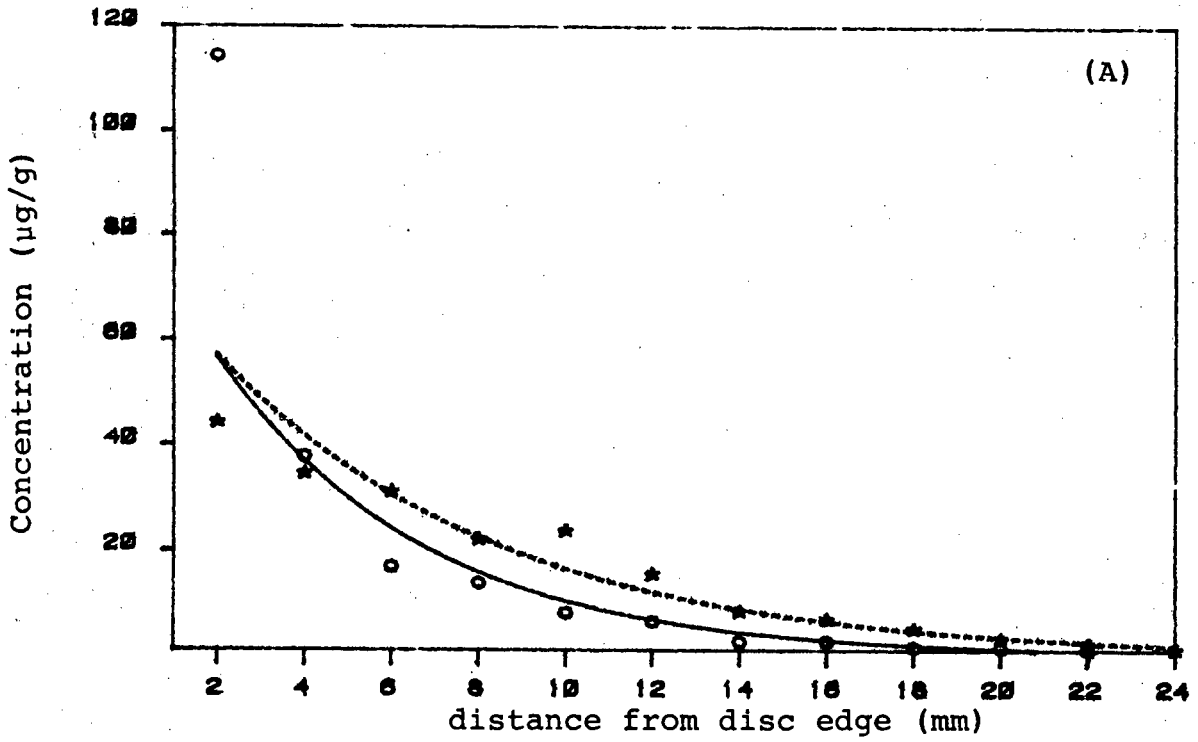
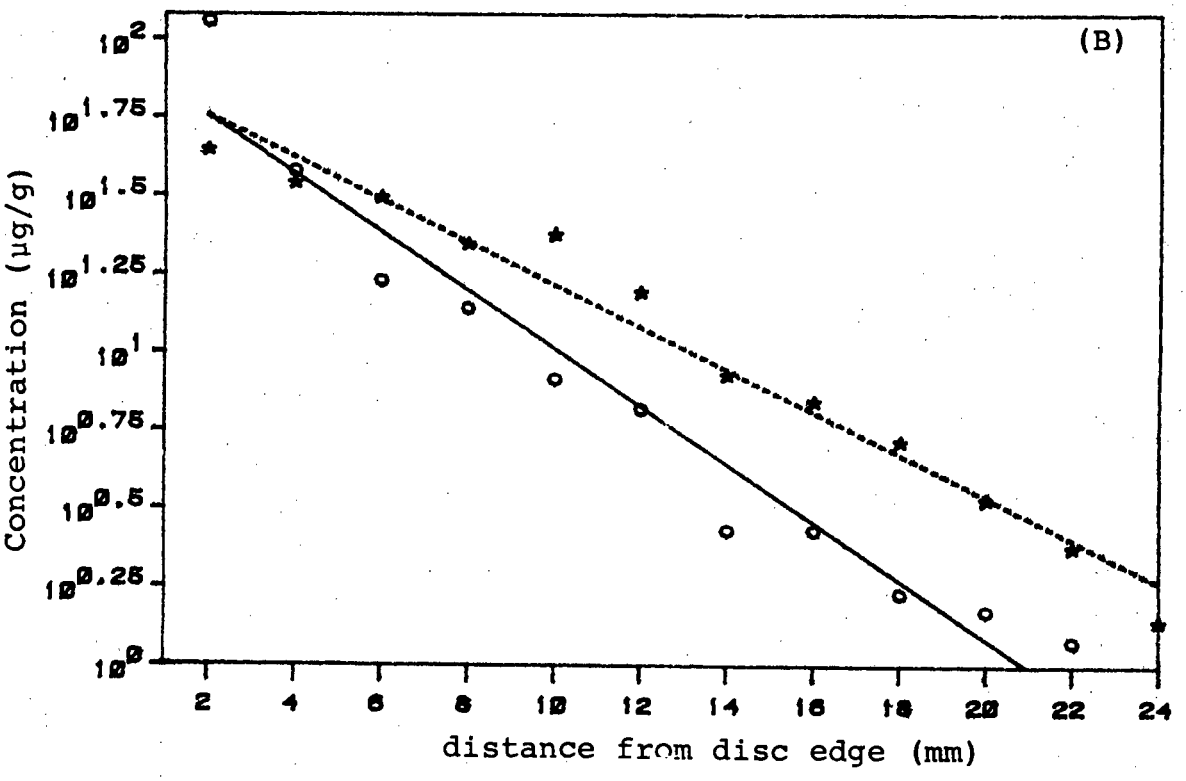


Fig. 2.3 Diffusion gradient for manganese chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



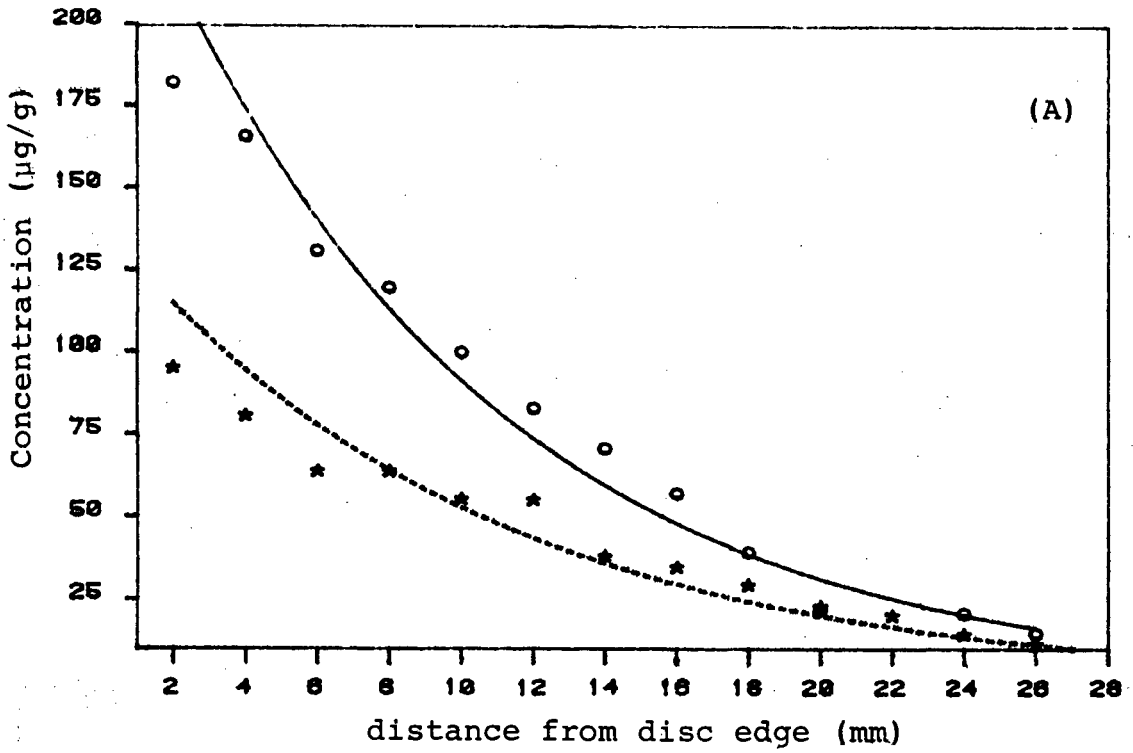
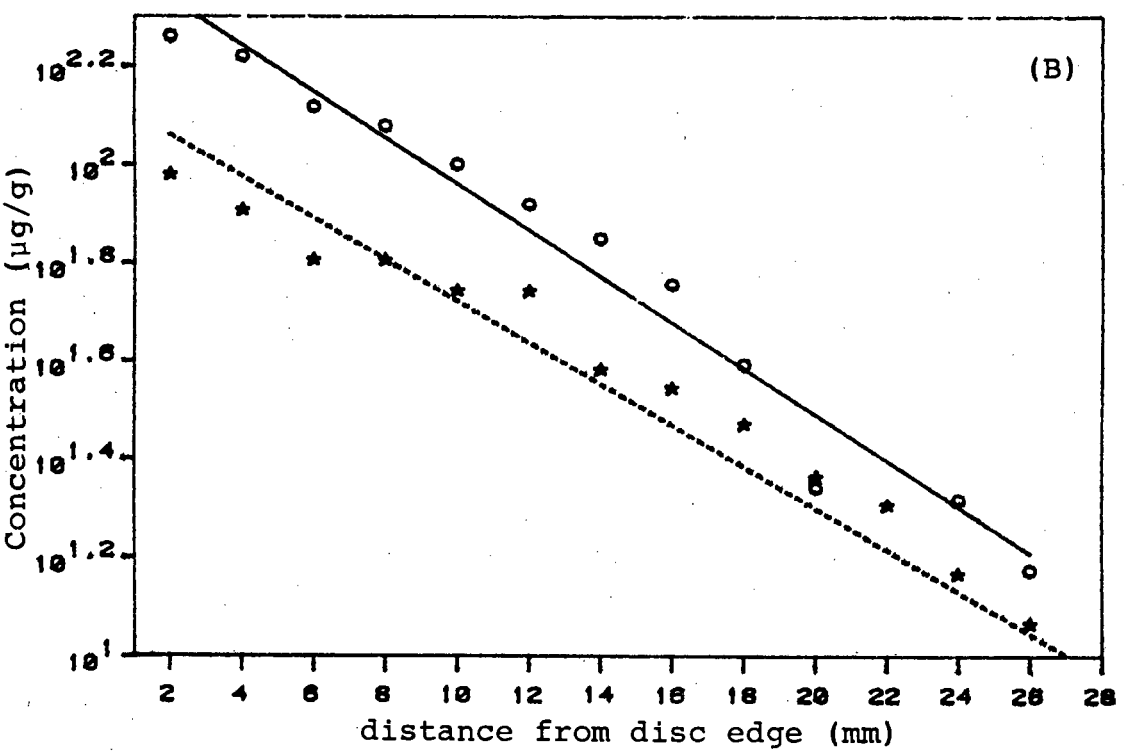


Fig. 2.4 Diffusion gradients for chromium trioxide
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



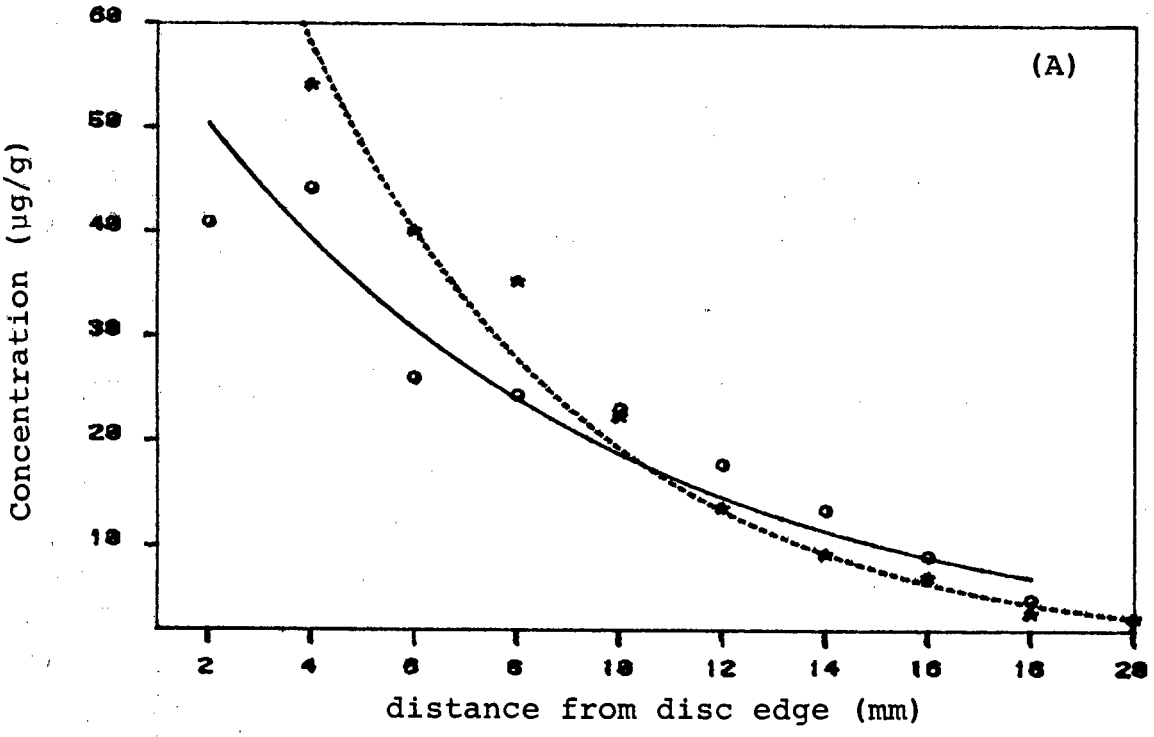
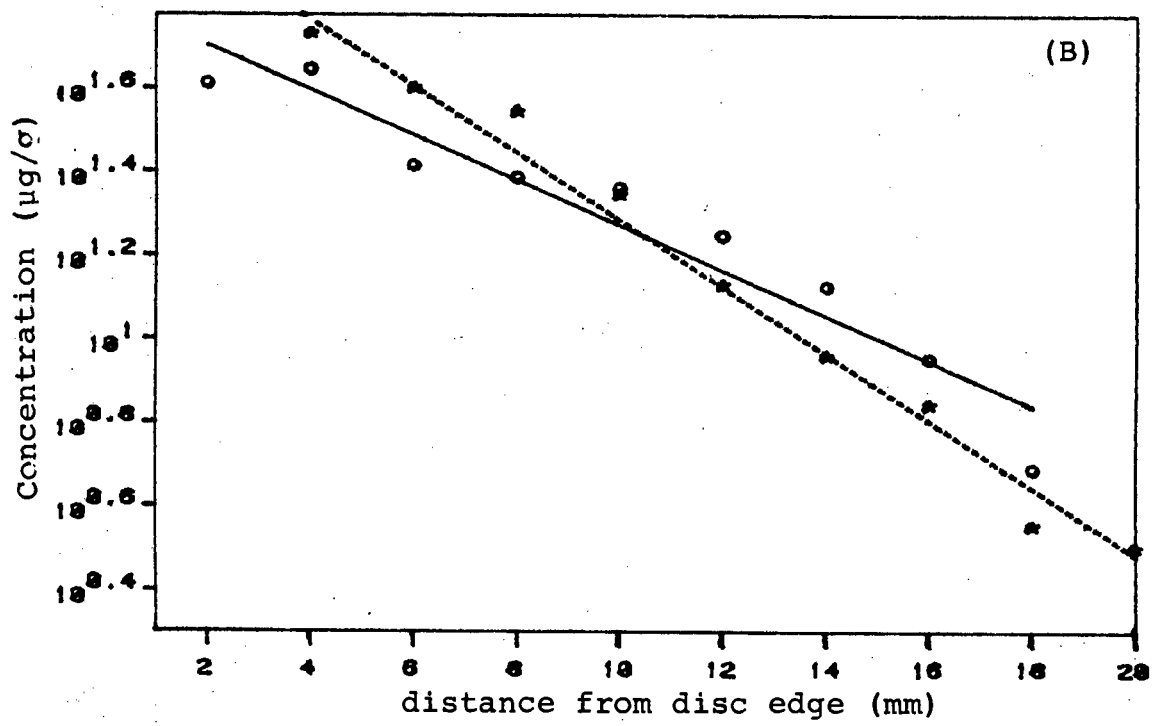


Fig. 2.5 Diffusion gradients for cadmium chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



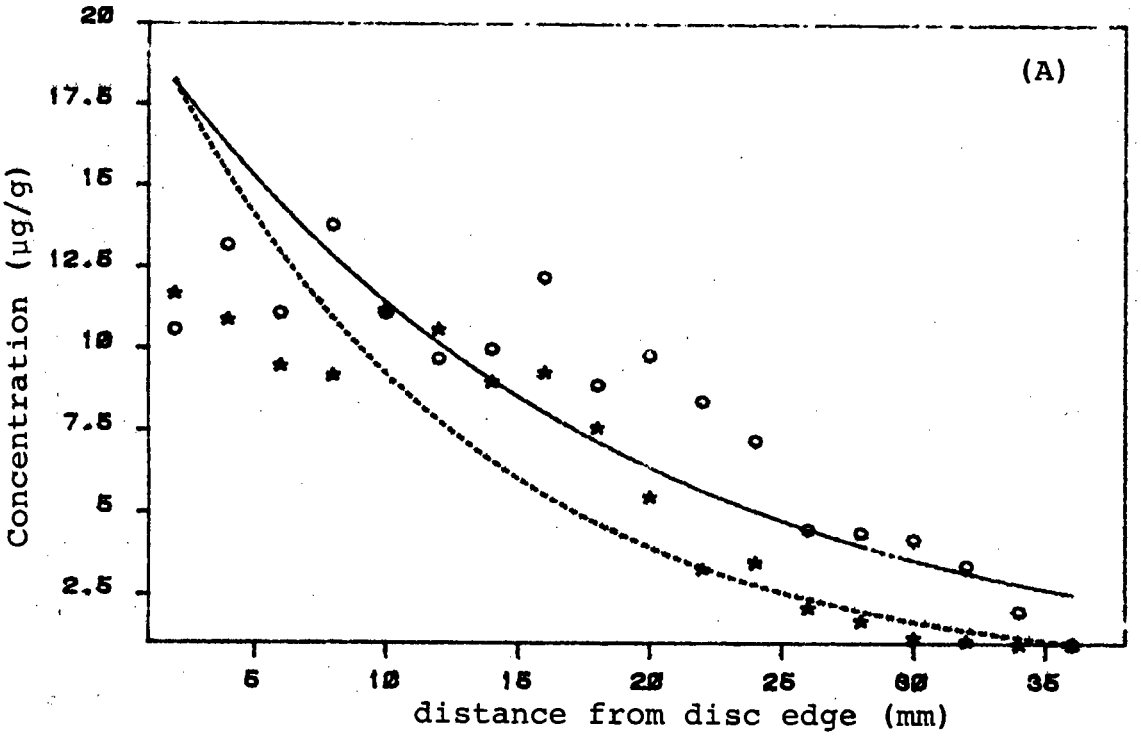
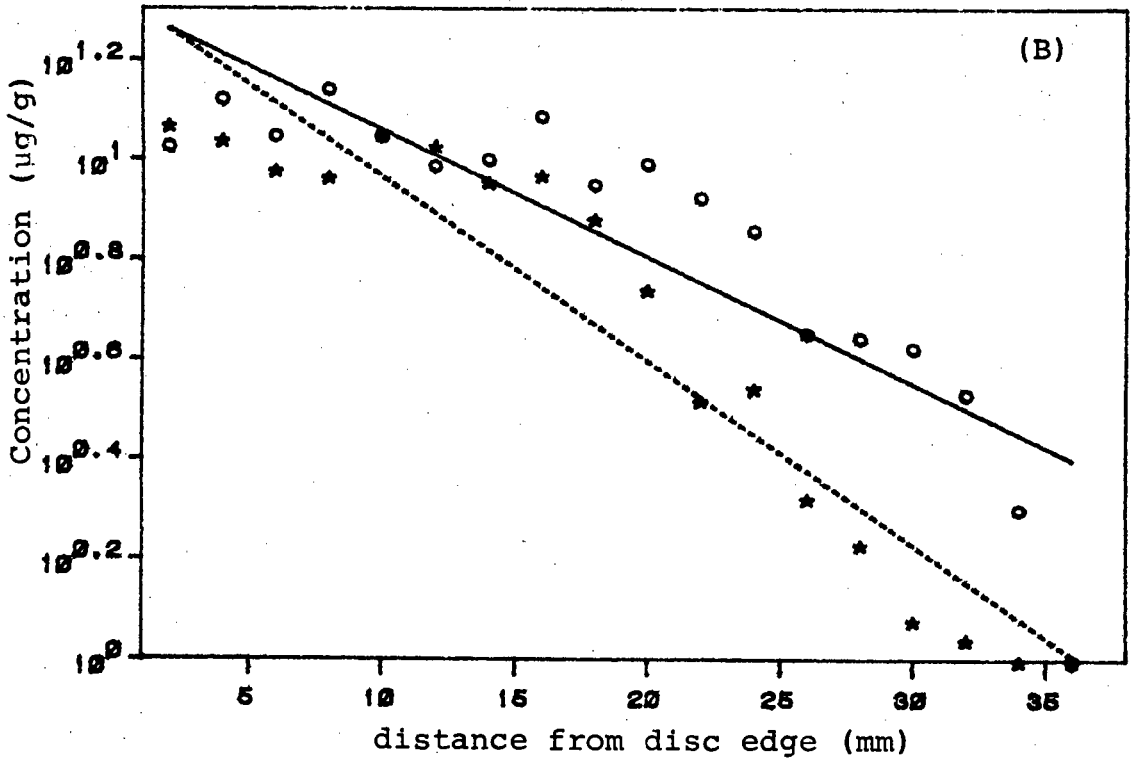


Fig. 2.6 Diffusion gradients for mercuric chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



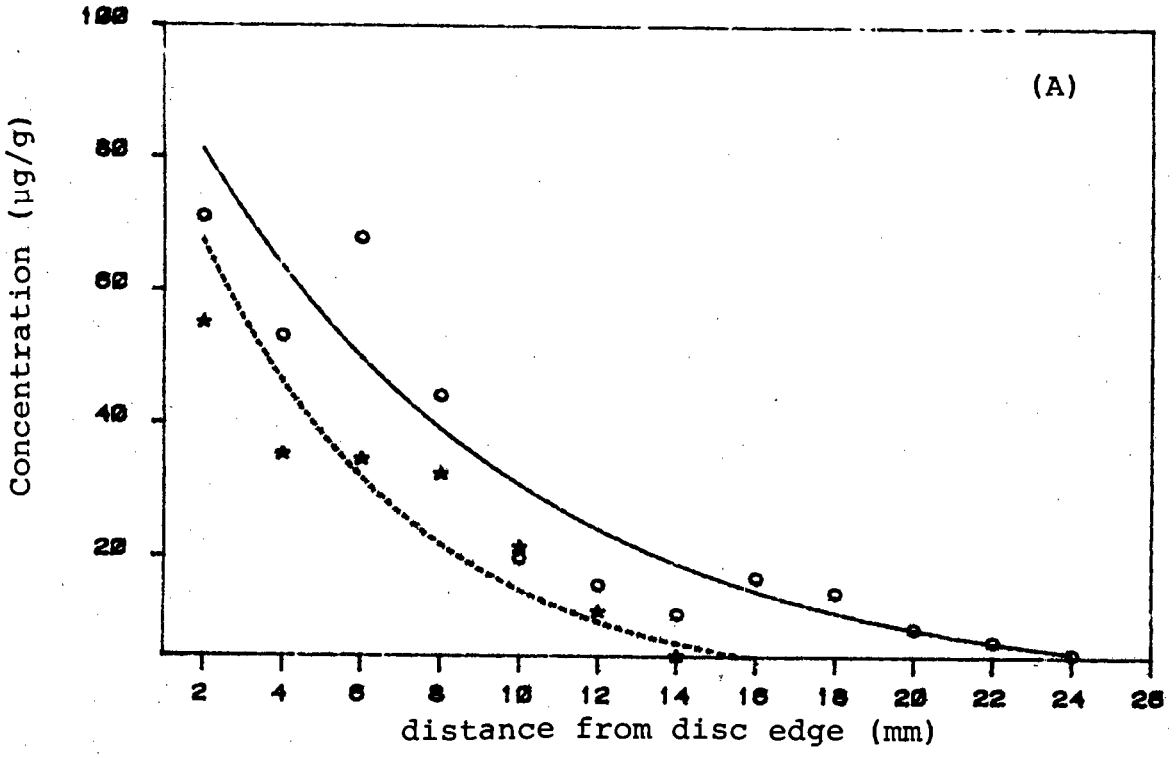
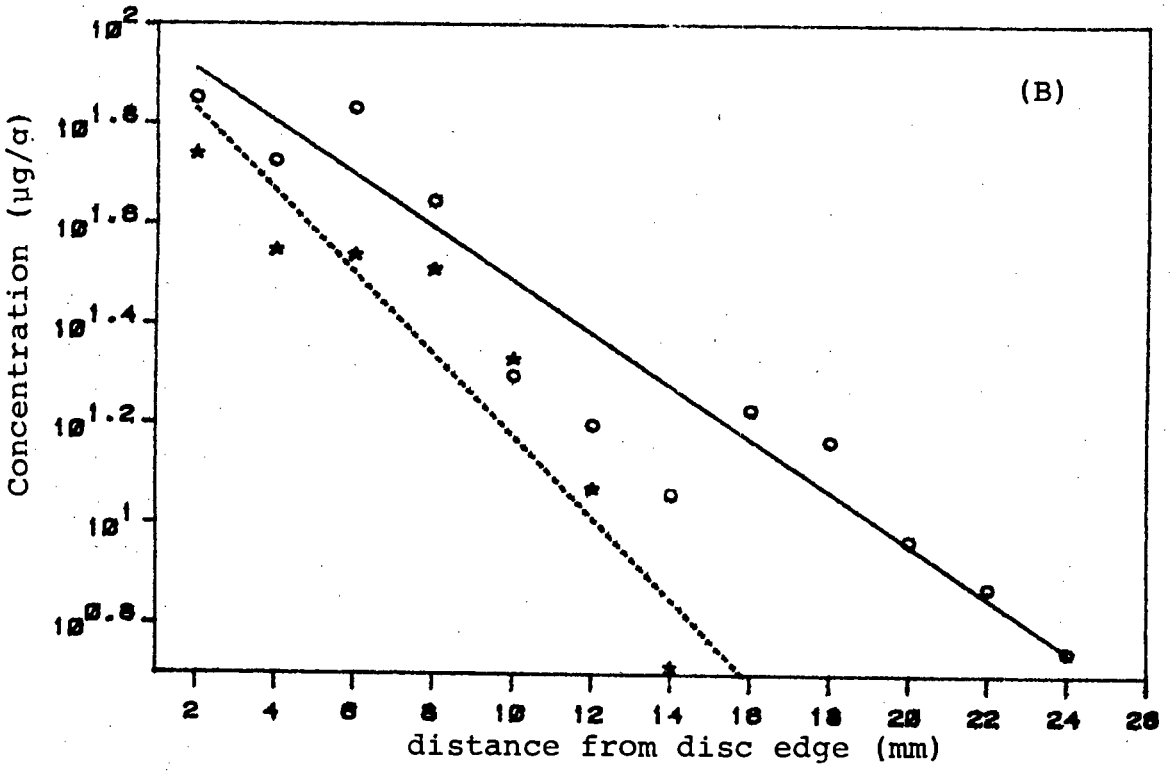


Fig. 2.7 Diffusion gradients for selenium dioxide
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



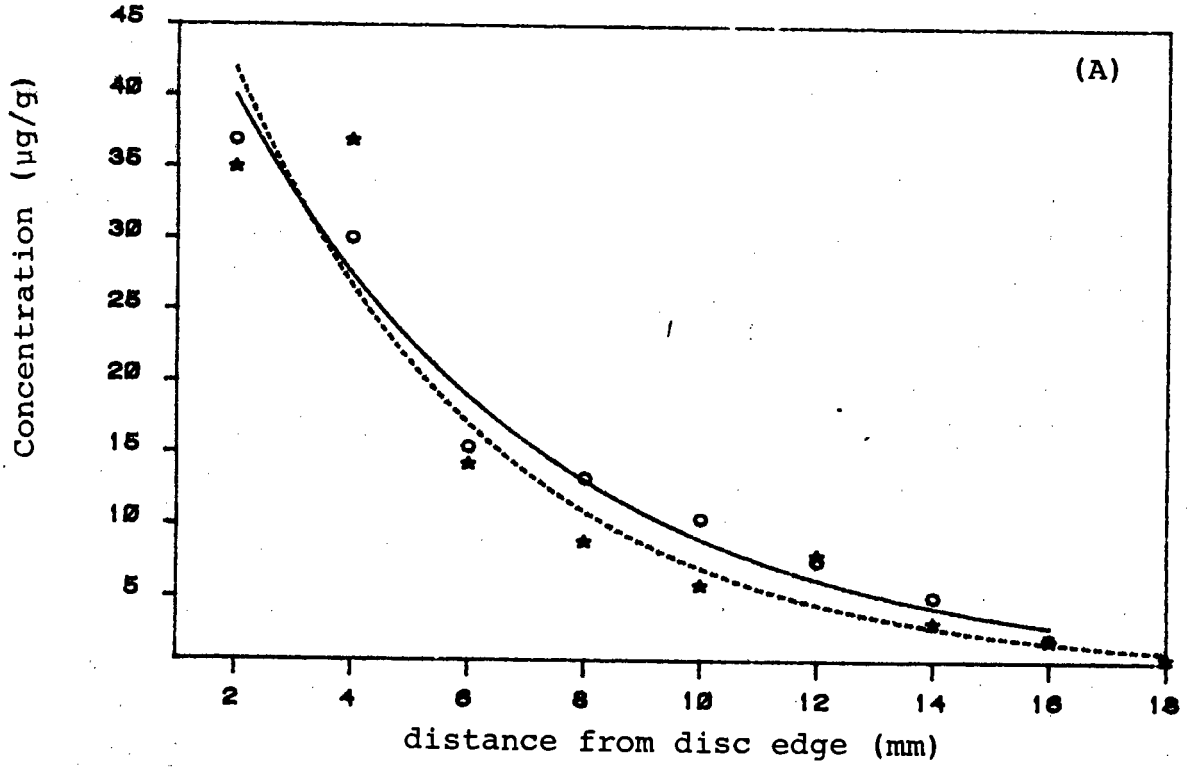
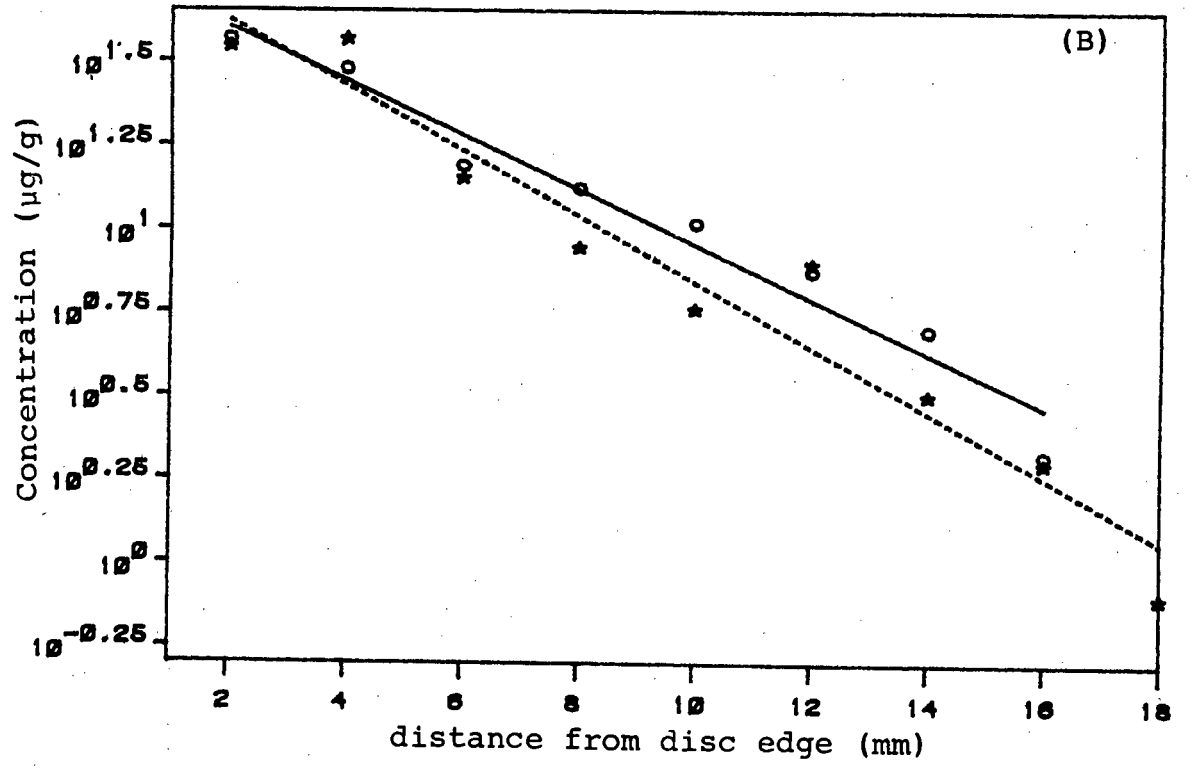


Fig. 2.8 Diffusion gradients for zinc chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



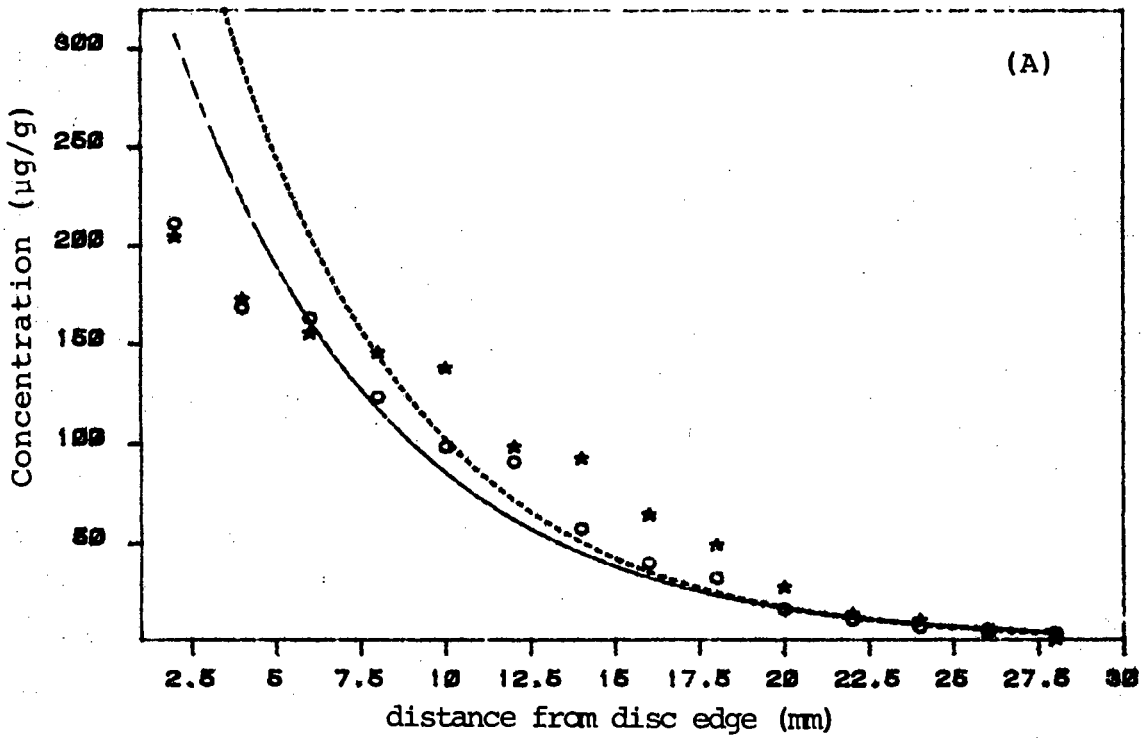
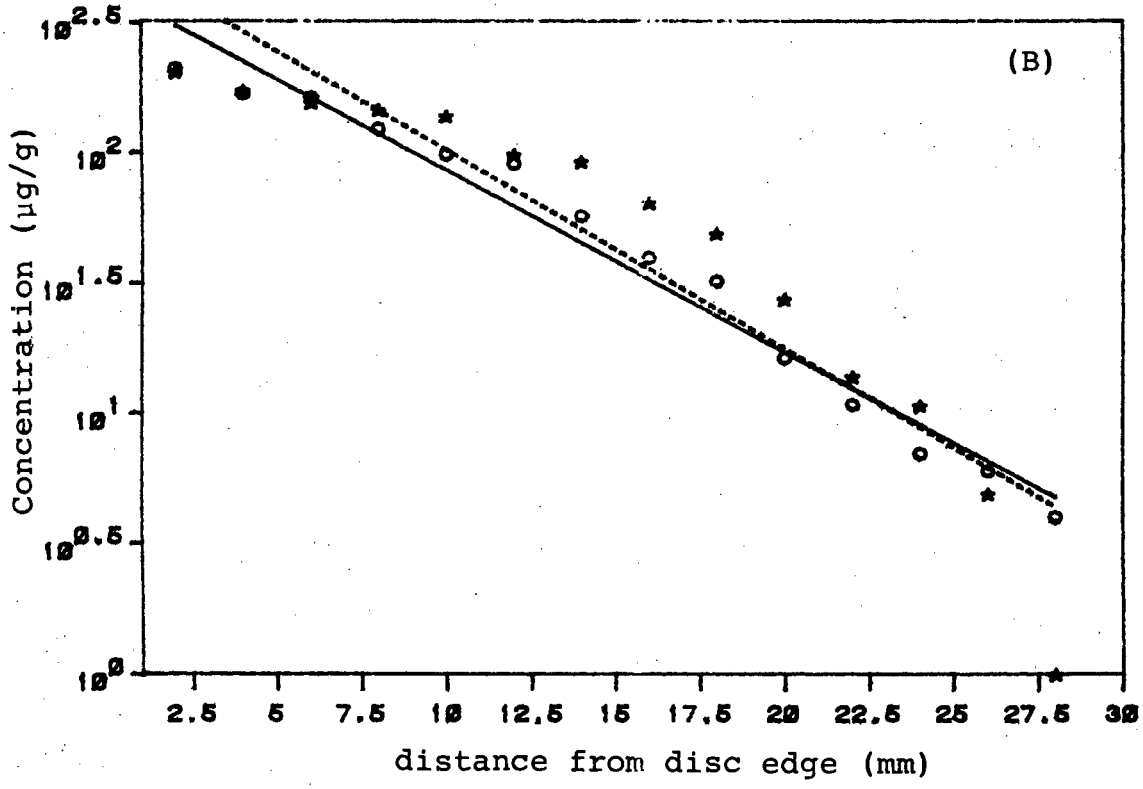


Fig. 2.9 Diffusion gradients for sodium arsenate
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



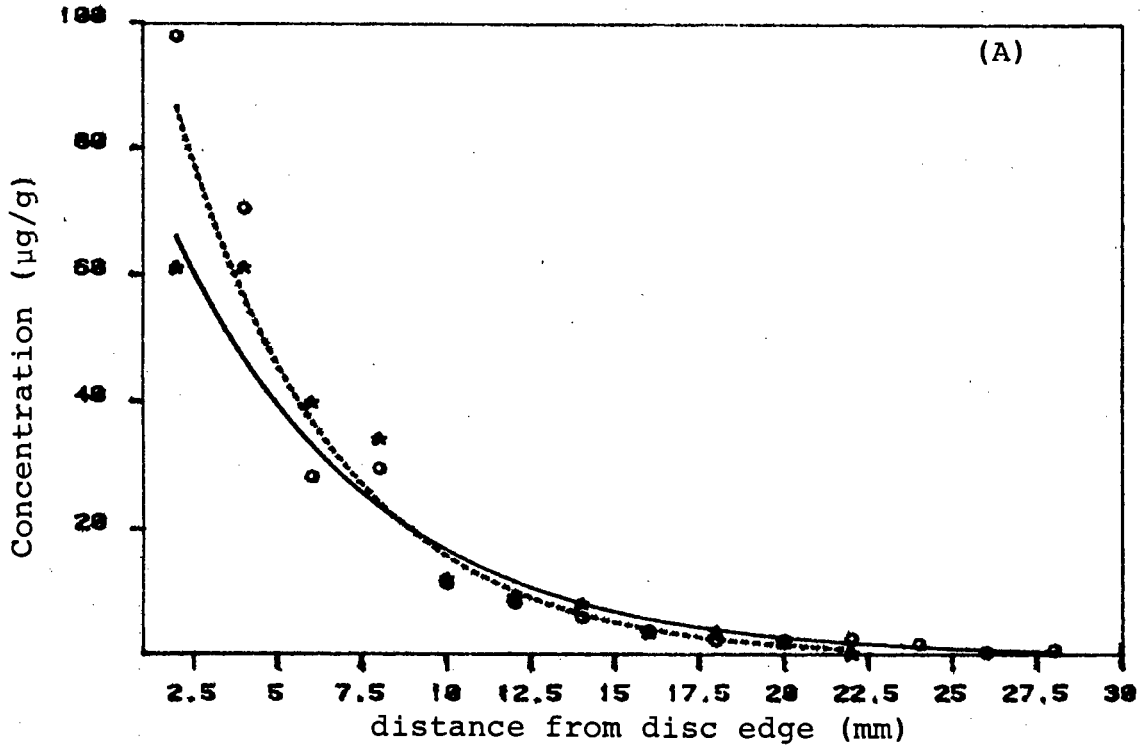
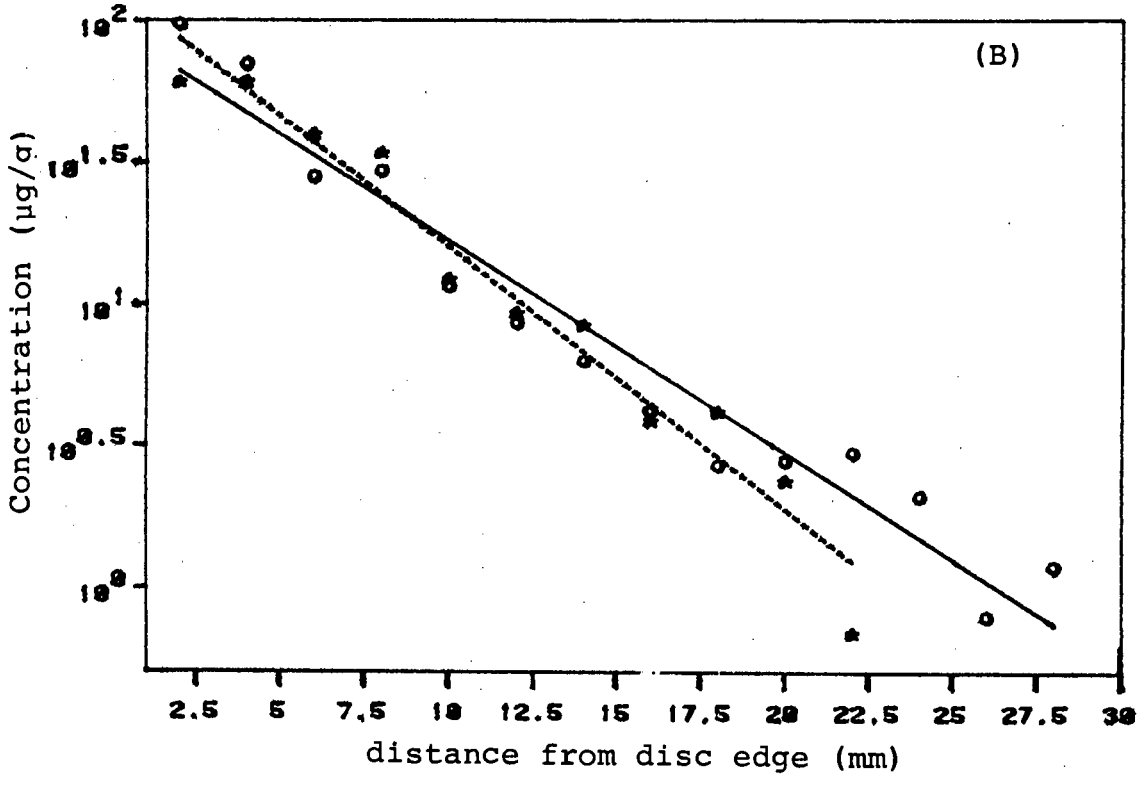


Fig. 2.10 Diffusion gradients for nickel chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



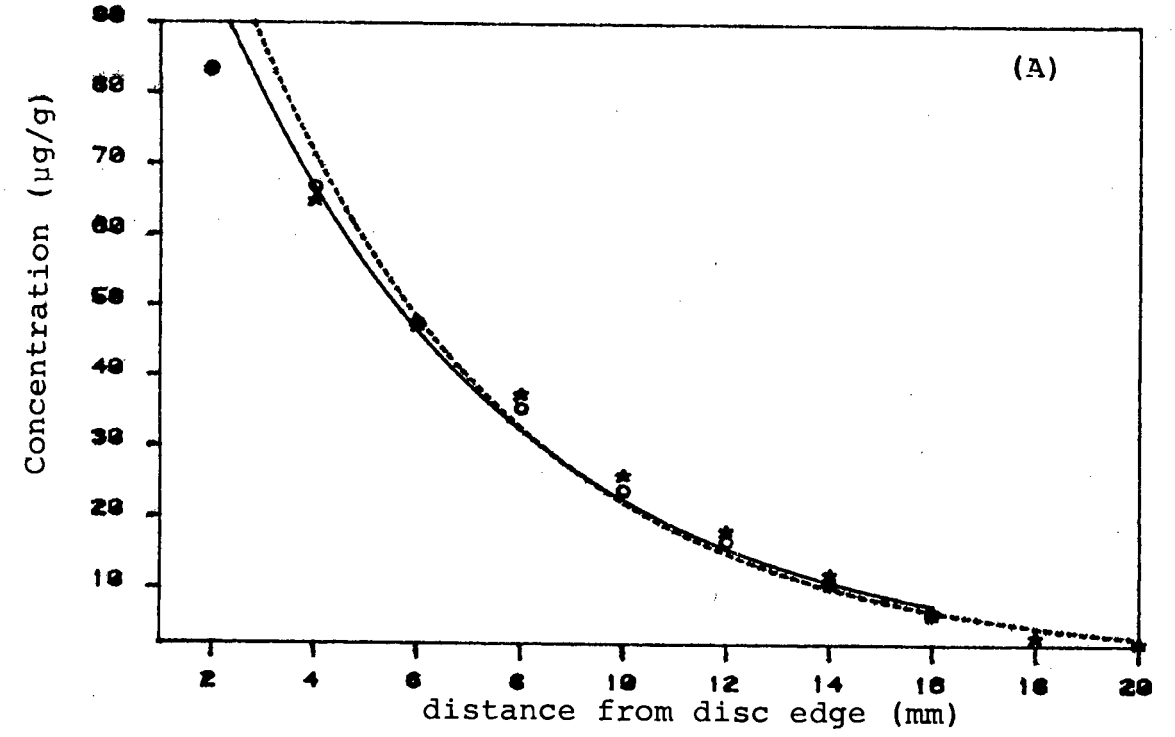
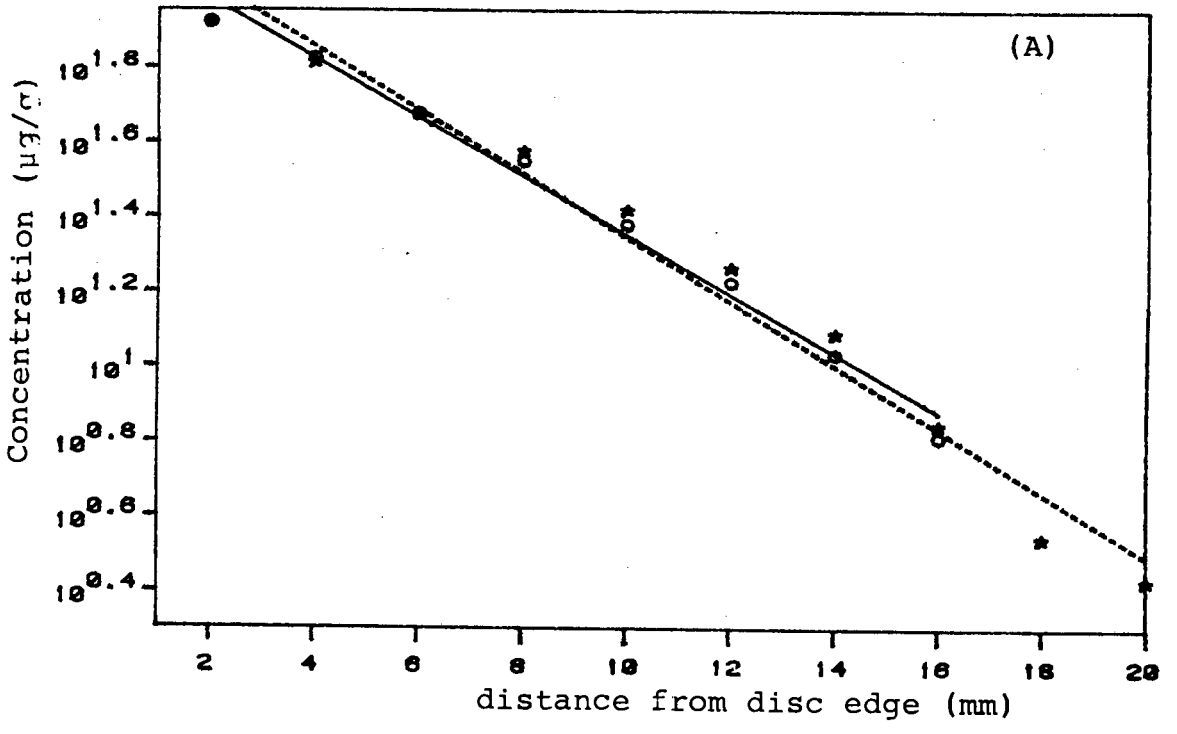


Fig. 2.11 Diffusion gradients for cobalt chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



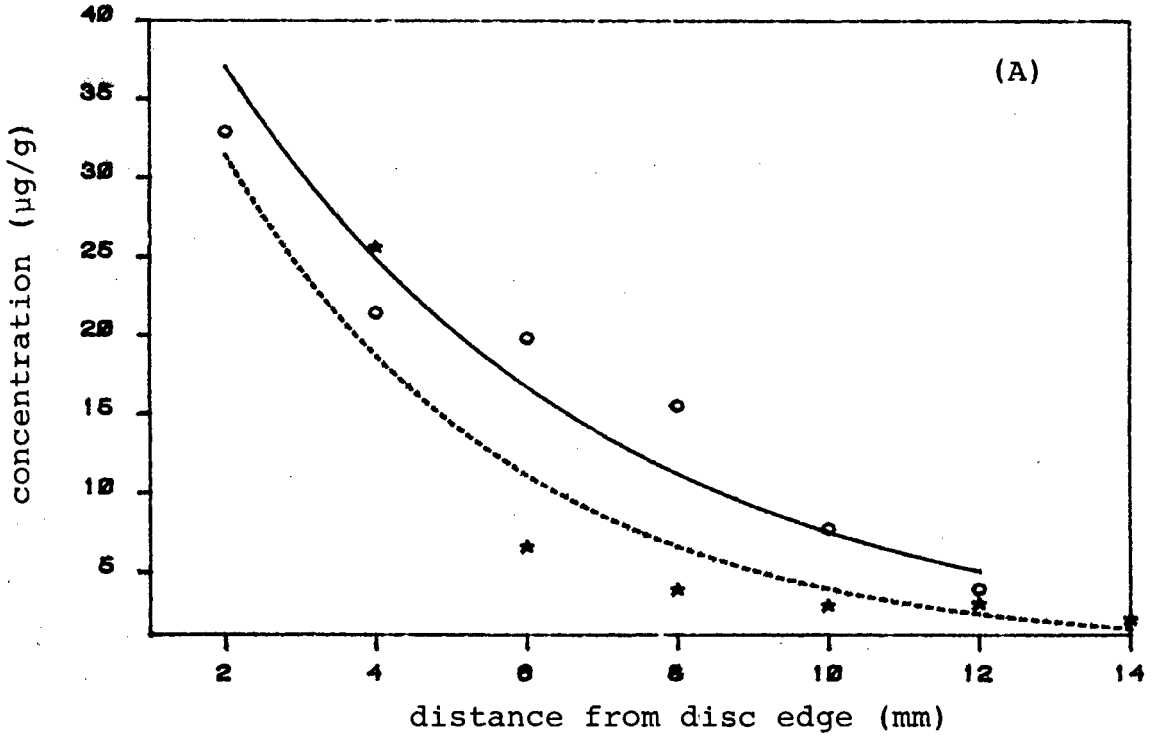
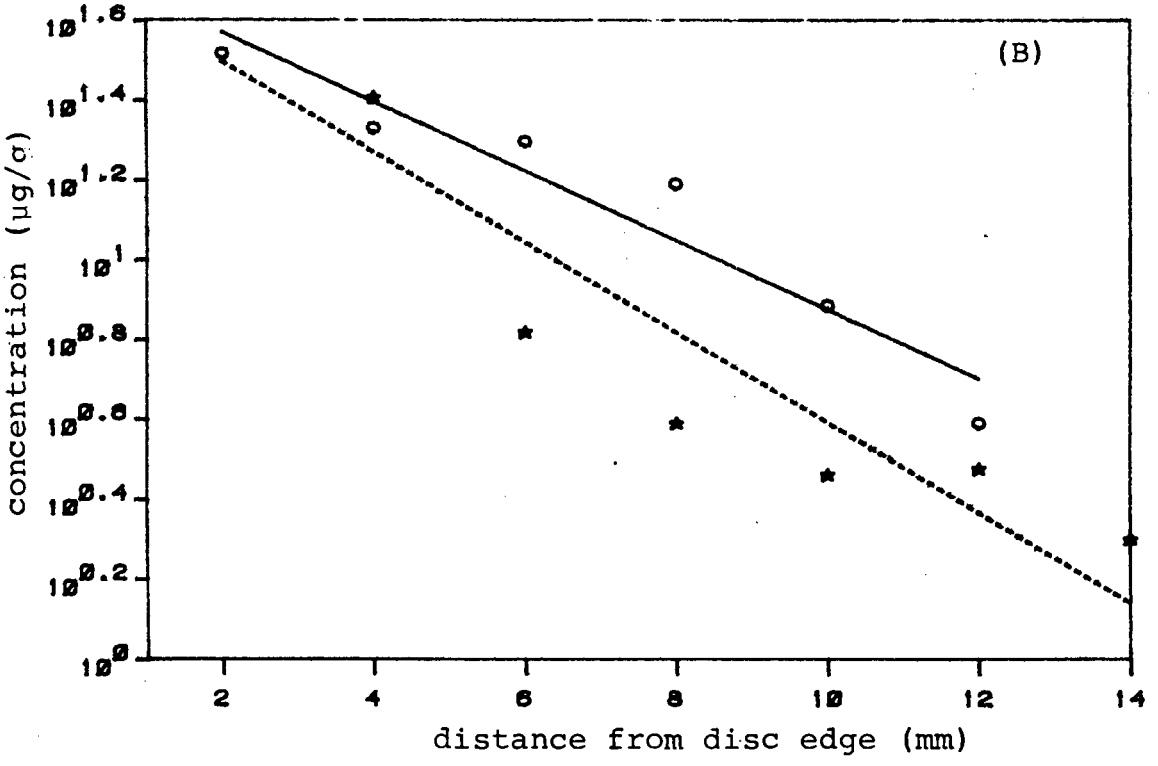
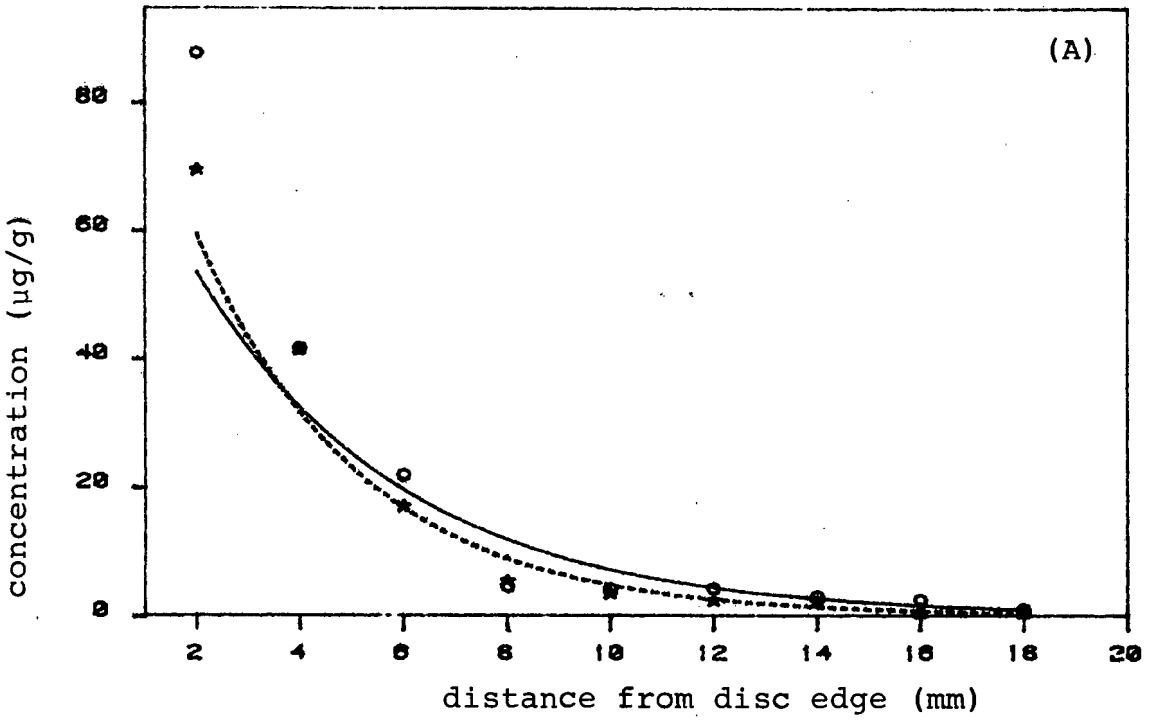


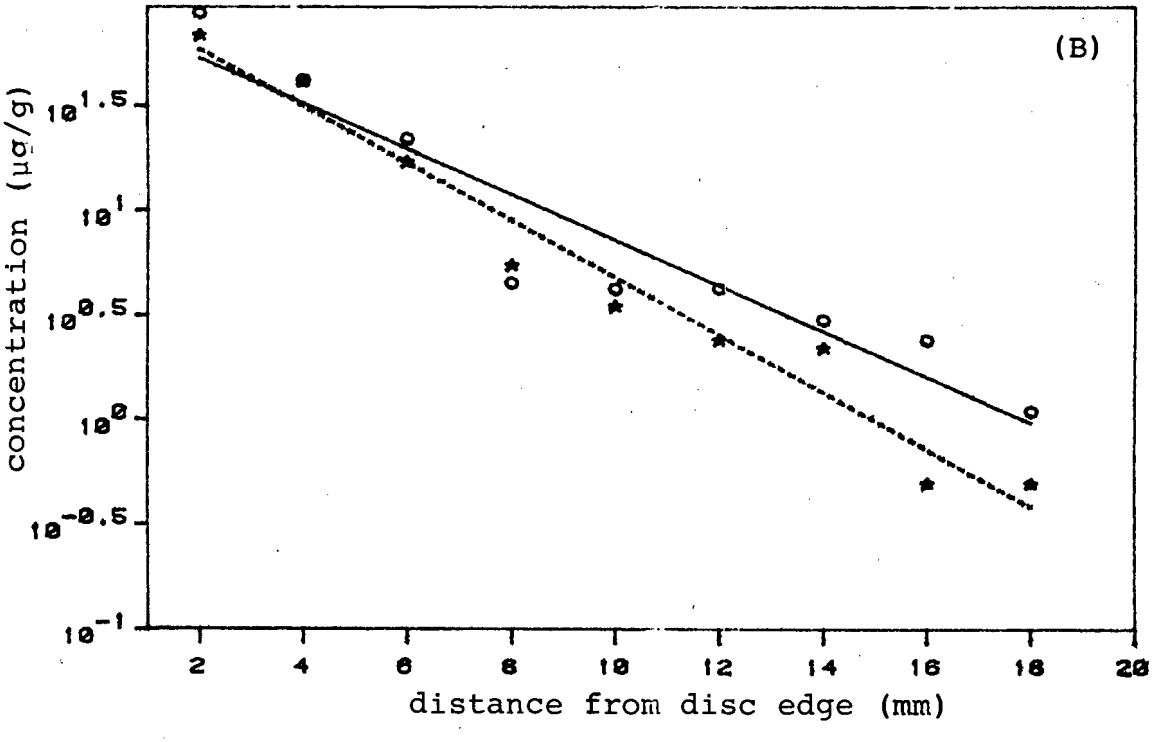
Fig. 2.12 Diffusion gradients for mercuric chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)





-Fig. 2.13 Diffusion gradients for lead chloride (Noble agar)

(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



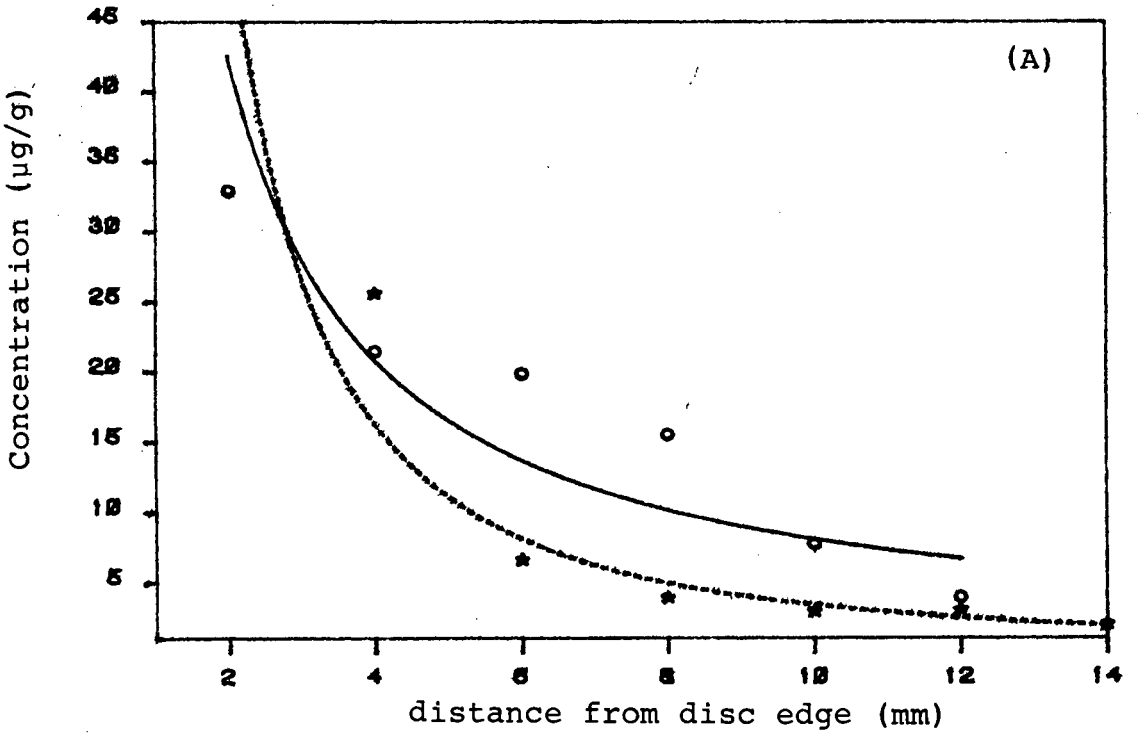
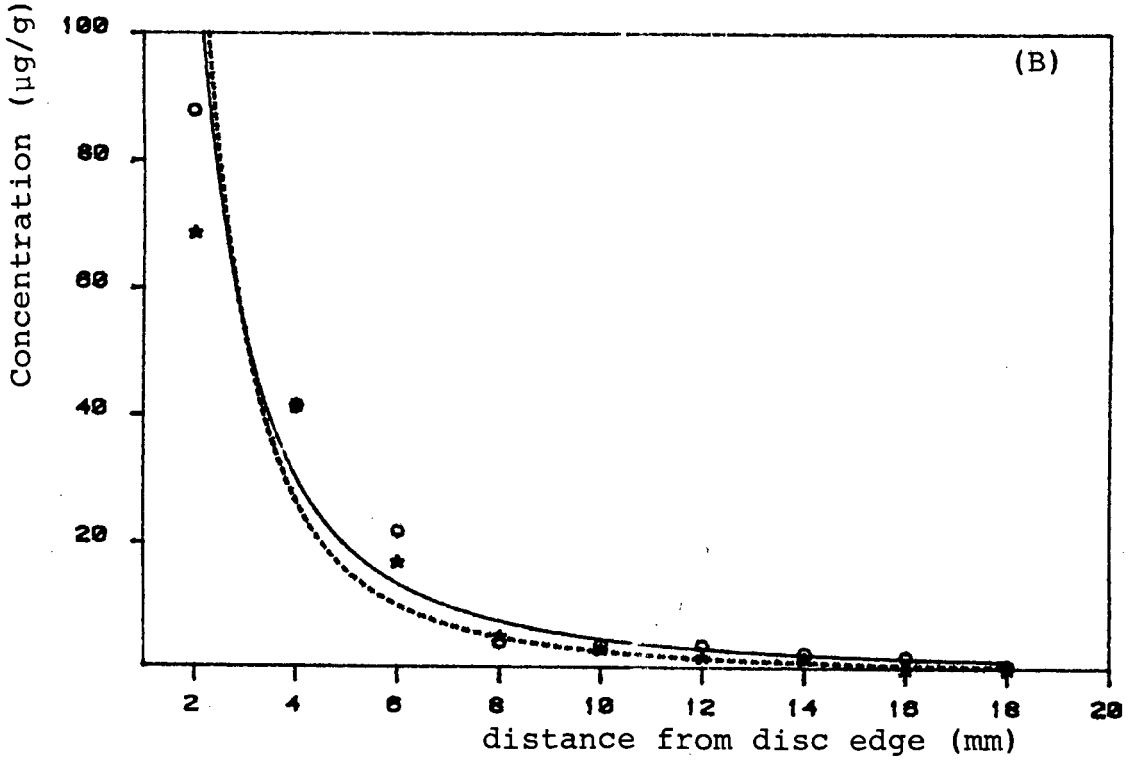


Fig. 2.14 Diffusion gradients : Power fit
(A) Mercuric chloride
(B) Lead chloride
(o = inoculated media; * = uninoculated media)



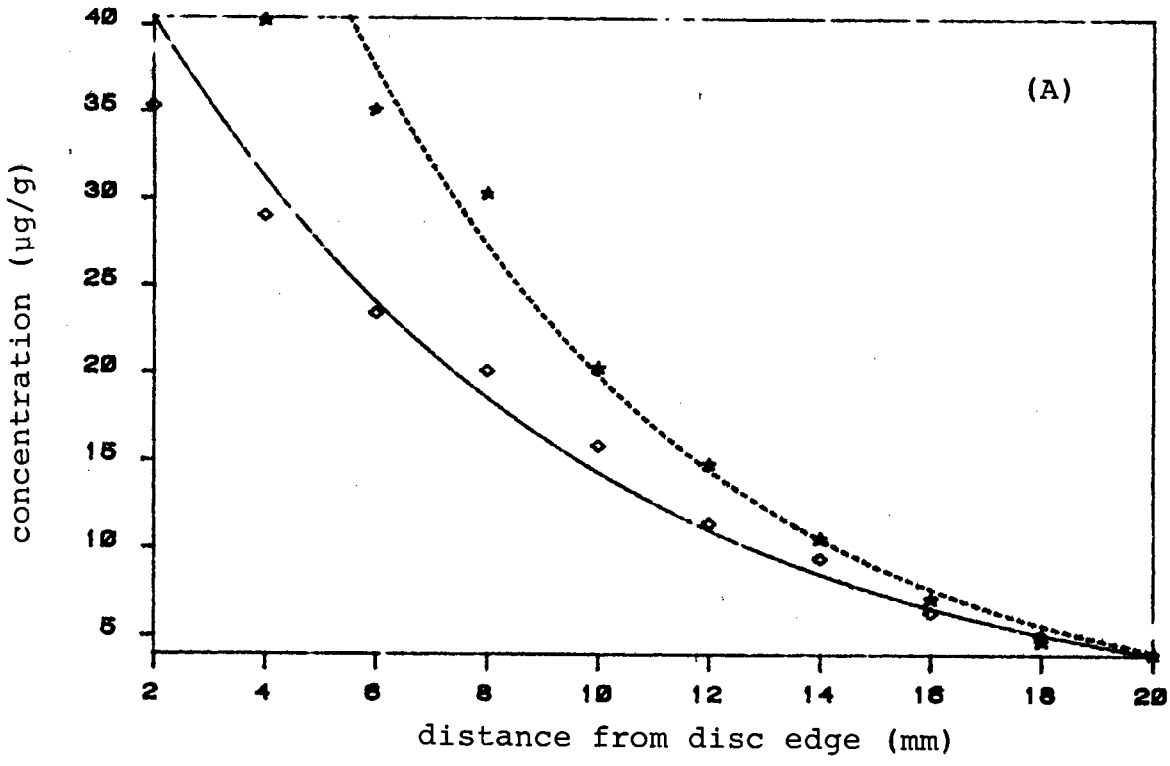
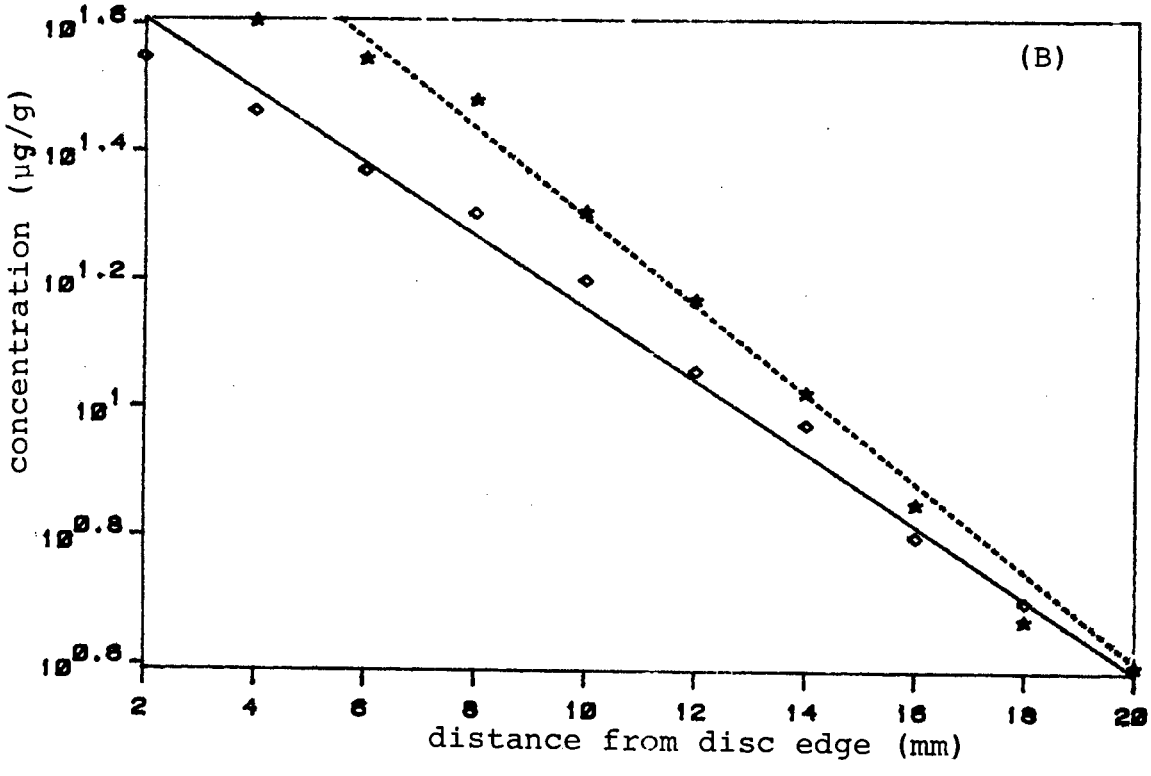


Fig. 2.15 Diffusion gradient (vertical) for cadmium chloride
(A) Exponential fit
(B) Log of concentration
(o = inoculated media; * = uninoculated media)



and placing the discs upon the plate. Table 2.2 lists the results of an experiment to determine the differences in zone sizes, if any, when metal containing discs were placed on inoculated media at set intervals. The time lapses between seeding the agar and placing the disc ranged from 0 to 240 minutes.

2.3.2 Diffusion gradients

Diffusion gradient graphs for each metal are shown in figures 2.2 to 2.13. These graphs were prepared from regression analysis of the data using the exponential fit formula

$$y = a \times e^{(b \times x)}$$

where y = metal concentration (µg/g)

x = distance from disc edge (mm)

a = the intercept

b = slope of the line

e = base to the natural logarithm

This fit appeared visually best for most of the metals. Comparison of exponential fit to other fit types by r^2 is shown in Table 2.3. From these results it was decided to draw power fit curves for lead chloride and mercuric chloride ($y = a \times x^b$) (Fig. 2.14).

Logarithmic slopes (log of metal concentration) were prepared for each metal compound under study. Statistical analysis of these demonstrated that at the 95% confidence level there was no significant

TABLE 2.2

Agar diffusion method; variations of zone sizes (mm) with time of application of metals to preseeded agar

Time (min)	$CuCl_2$	$CdCl_2$	CrO_3	$CoCl_2$	$NiCl_2$	$ZnCl_2$	$HgCl_2$	$PbCl_2$
0	18,5	23,7	26,9	24,9	17,8	24,4	30,2	15,6
30	16,7	22,5	25,7	24,2	18,0	23,5	31,2	15,4
60	15,8	20,8	23,8	24,6	20,0	23,0	30,6	15,7
120	16,0	20,5	23,0	24,1	19,2	21,0	28,1	15,7
240	16,0	20,4	22,3	19,0	16,9	21,8	27,0	14,2

TABLE 2.3

Regression analysis of diffusion gradient data

METAL COMPOUND	Exponential fit - r^2 (a)	(b)	Best fit - r^2 (a)	(b)
Lead chloride	0,8961	0,9648	0,9711 P	0,9648 E
Mercuric chloride	0,9598	0,9347	0,9791 LI	0,9776 P
Cadmium chloride	0,9222	0,9908	0,9222 E	0,9908 E
Nickel chloride	0,9452	0,9563	0,9452 E	0,9563 E
Chromium trioxide	0,9489	0,9137	0,9549 LO	0,9653 L
Zinc chloride	0,9588	0,9424	0,9702 LO	0,9424 E
Copper chloride	0,9472	0,9719	0,9472 E	0,9719 E
Cobalt chloride	0,9772	0,9893	0,9772 E	0,9893 E
Manganese chloride	0,8345	0,9706	0,8664 P	0,9706 E
Methyl mercuric chloride	0,7572	0,8947	0,8328 LI	0,9094 LI
Selenium dioxide	0,8410	0,9089	0,8410 E	0,9219 LI
Sodium arsenate	0,9710	0,8577	0,9710 E	0,9710 LI

P = Power LI = Linear E = Exponential LO = Log (a) = inoculated media (b)=uninoculated media

difference between the slopes of inoculated and uninoculated media. The only exception to this finding was for chromium trioxide where the slopes differed significantly at the 99,9% confidence level. Comparisons of exponential slopes, using the F. statistic were carried out for cadmium, lead, zinc, nickel, chromium, cobalt and mercury to copper. The results demonstrated that the exponential rate of diffusion is metal specific although the slopes of nickel and copper did not differ significantly at the 95% confidence level.

2.3.3 MIC measurements

The zone of inhibition of bacterial growth was measured in millimetres from the edge of the disc to the inner edge of the normal growth zone. The MIC was calculated from the diffusion gradient curve and expressed as µg/g metal in the agar. These results are summarised in Table 2.4.

In the case of lead and manganese, no zones of inhibition of bacterial growth were visible. The lead diffusion gradient (Fig.2.13) indicated that this metal had diffused poorly through the agar. The manganese diffusion characteristics (Fig. 2.3) were also poor, and it was concluded that the absence of a zone of inhibition was due either to the non-toxic nature of the element or to its

TABLE 2.4

MIC values for the study compounds

Metal Compound	Zone (mm)	MIC (µg/g)
Zinc chloride	4,0	37,0
Sodium arsenate	10,1	118,0
Manganese chloride[1]	No zone	115,0
Manganese chloride[2]		
Nickel chloride	2,7	88,0
Chromium trioxide	6,7	127,0
Methylmercuric-chloride	25,0	5,5
Mercuric chloride	13,3	3,5
Selenium dioxide	9,2	30,0
Lead chloride[1]	No zone	-
Lead chloride[2]	4,3	38,0
Cobalt chloride	5,9	50,0
Cadmium chloride	9,7	29,5
Copper chloride	2,7	146,0

1 = Nutrient agar
2 = 1,2% Noble agar media

diffusion characteristics. The experiments were repeated for lead and manganese using various other media (Table 2.5).

Optimum results were obtained for lead when 0,1% tryptone and 0,5% sodium chloride were added to Noble agar or Biolab High Purity agar and the inoculum was 0,5 ml of an overnight culture of E. coli in 5 ml of 1% tryptone water per 100 ml media. This modified media yielded a good diffusion gradient for lead with visible zones of bacterial growth. The diffusion gradient characteristics for manganese improved slightly but no zone of inhibition was visible, confirming that this element is non-toxic at the concentrations tested (Fig. 2.16; 2,17).

2.3.4 Identification of heterotrophic sediment bacteria

From the results of the API 20E system of identification and also Gram stain morphology it was possible to classify 26 strains of Gram negative bacteria and 17 strains of Gram positive bacteria isolated from the six sites in the Swartkops River. The composition of the species is listed in Table 2.6.

2.3.5 Analysis of zone sizes and metal toxicity

For each metal compound zone size measurements were

TABLE 2.5

Composition of test media for lead and manganese diffusion trials

Media No	Agar Make	% Agar	% Tryptone	% NaCl	% Other Nutrients
1	Oxoid	1,5	-	0,5	Peptone 0,5; Yeast Extract 0,2; Lablemco 0,1
2	Noble	1,2	-	-	-
3	Noble	1,2	0,5	0,5	-
4	Noble	1,2	0,25	0,5	-
5	Noble	1,2	0,1	0,5	-
6	Biolab*	1,2	-	-	-
7	Biolab*	1,2	0,5	0,5	-
8	Biolab*	1,2	0,25	0,5	-
9	Biolab*	1,2	0,1	0,5	-

* High Purity

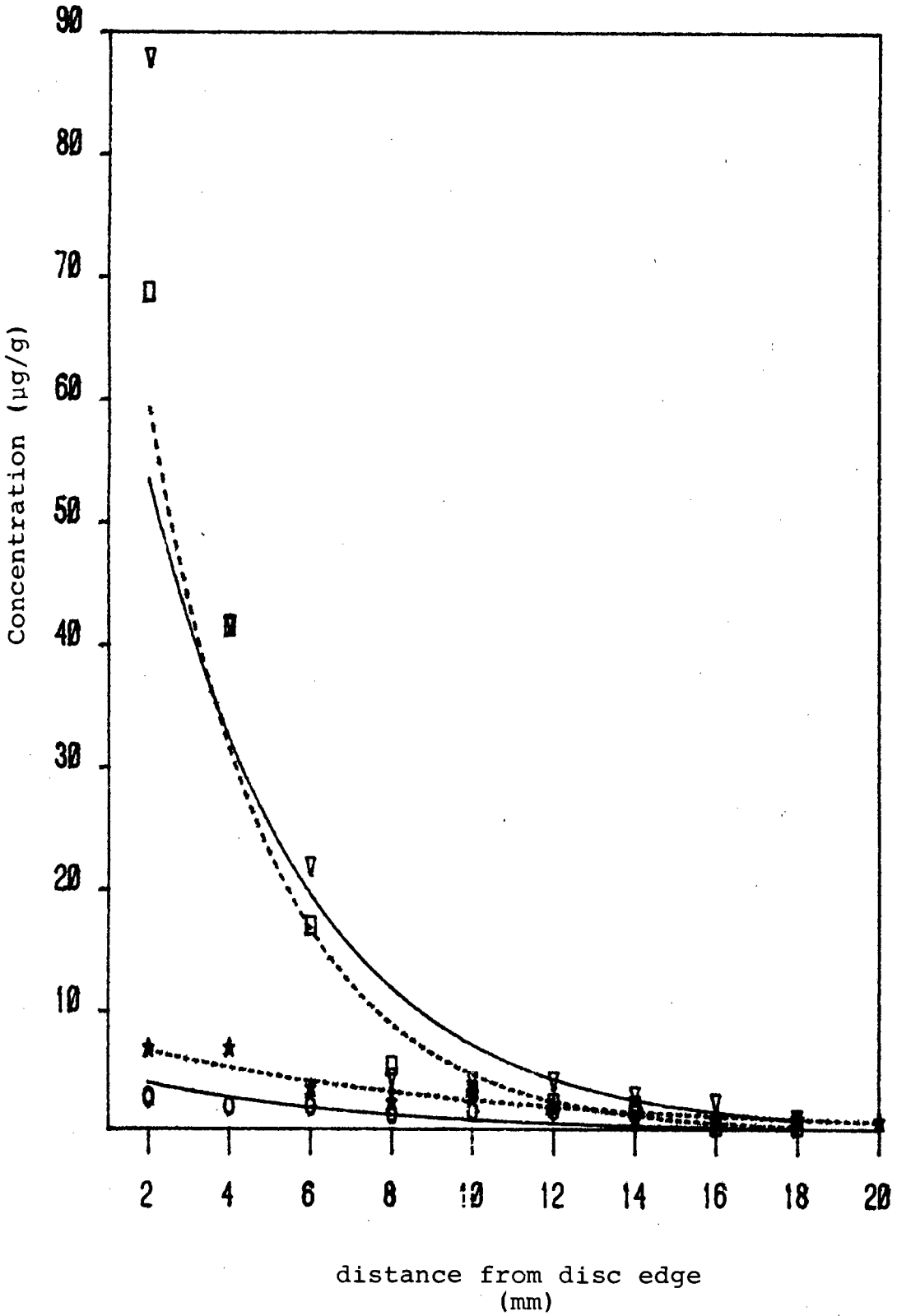


Fig. 2.16 Comparison of diffusion rate of lead in Noble agar media and nutrient agar

□---uninoculated Noble agar media
▽——inoculated Noble agar media
*---uninoculated nutrient agar
o——inoculated nutrient agar

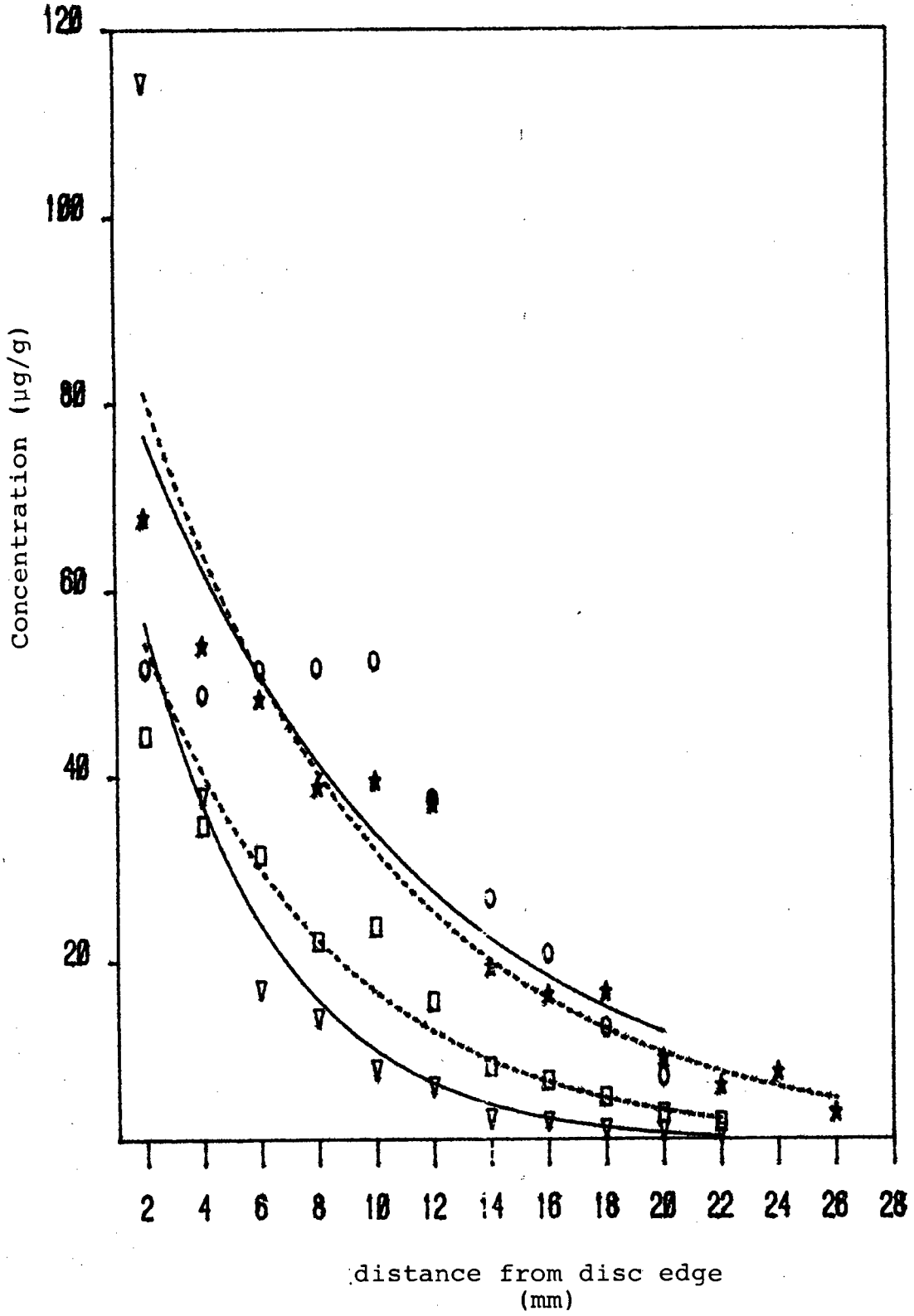


Fig. 2.17 Comparison of diffusion rate of manganese in Noble agar media and nutrient agar

▽---uninoculated Noble agar media
□——inoculated Noble agar media
*---uninoculated nutrient agar
o——inoculated nutrient-agar

TABLE 2.6

Species composition of bacteria from Swartkops River sediments

Site	Gram positive bacteria	Gram negative bacteria
1	*Bacillus* sp. (ST/O)(3) *Bacillus* sp. (ST/R)	*Vibrio alginolyticus* (2) *Escherichia coli*
2	*Bacillus* sp. (T/O) *Bacillus* sp. (C/O) *Bacillus* sp. (T/R)	*E. coli* (2) *Enterobacter cloacae* *Pseudomonas* sp.
3	*Bacillus* sp. (T/O) *Bacillus* sp. (T/R)	*E. coli* *Aeromonas* sp. *Pseudomonas fluorescens* *Citrobacter freundii*
4	*Bacillus* sp.(T/O) *Bacillus* sp. (ST/O)(2)	*Klebsiella* sp. *Pseudomonas putrefaciens* (2) *Pseudomonas* sp. *Acinetobacter* sp. Unidentified sp.
5	*Bacillus* sp.(ST/O) *Bacillus* sp.(T/O) *Bacillus* sp.(C/O)	*Aeromonas hydrophila* (2) *E. coli* *Klebsiella oxytoca* Unidentified sp.
6	*Bacillus* sp.(ST/O) *Bacillus* sp.(T/R)	*C. freundii* *A. hydrophila* *E. cloacae* *Acinetobacter* sp.

Key: T = Terminal) spore position
ST = Sub-terminal)
C = Central)

R = Round) spore shape
O = Oval)

obtained to each bacterial strain. The mean ($\Sigma X_i^n/n-1$) Median, variance ($\Sigma_i^n(X_i-\bar{X})^2/n-1$) and standard deviation ($/\overline{s^2}$) were calculated for both the Gram positive and the Gram negative bacteria. Normal distribution could not be assumed due to the differences in sample size and species. Therefore the Mann-Whitney test statistic was applied to the results (Table 2.7).

Mann-Whitney Statistic -

$$U = R_1 - \frac{n_1 (n_1 + 1)}{2}$$

where R_1 = Σ ranks of group

n = number of samples

Greater toxicity to Gram positive bacteria was found for five metal compounds at the 95% confidence level and for three metal compounds at the 90% confidence level. In the case of three metals, copper, selenium and chromium, the Gram negative bacteria proved more susceptible. Zinc was the only metal that did not exert a significant difference in toxicity to either groups of bacteria.

2.4 DISCUSSION

Disc diffusion has been used routinely for many years to determine antibiotic susceptibility of bacteria (Bauer *et al* 1965; Ericsson & Sherris 1971). By standardising certain

TABLE 2.7

Relationships of zone size variations between Gram positive and Gram negative bacteria

	(1)		(2)		(3)		(4)		(5)		(6)		(7)		(8)		(9)		(10)		(11)		(12)	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Mean	13,01	8,95	12,76	9,48	5,97	3,98	1,47	5,52	10,05	5,81	3,22	0,47	7,23	6,15	4,29	3,26	5,97	5,68	3,80	3,84	0,44	1,71	1,55	7,91
Median	13,10	8,15	12,50	9,60	6,10	3,90	0,10	6,40	10,75	5,80	3,30	0,10	7,35	5,00	4,35	3,10	5,90	5,40	3,70	3,30	0,10	1,10	0,10	6,85
Variance	13,68	8,01	11,67	5,70	2,45	1,51	4,73	23,66	9,87	5,95	8,56	0,80	6,46	7,46	3,74	3,29	0,70	4,89	3,00	7,57	0,89	4,72	8,93	30,21
Standard Deviation	3,69	2,83	3,42	2,38	1,56	1,23	2,17	4,86	3,40	2,44	2,92	0,90	2,54	2,73	1,93	1,81	0,84	2,20	1,73	2,75	0,94	2,17	2,99	5,50
Mann-Whitney Test Statistic	355,0		345,0		377,5		107,0		369,0		330,5		285,0		291,5		293,5		255,0		126,0		49,5	
Level of Significance	<0,001		0,002		<0,001		0,004		<0,001		0,001		0,08		0,06		0,07		0,40		0,009		<0,001	

(1) Ethyl Mercuric Chloride; (2) Methyl Mercuric Chloride; (3) Mercuric Chloride; (4) Selenium Dioxide; (5) Monosodium Methane Arsonate; (6) Sodium Arsenate; (7) Cadmium Chloride;

(8) Nickel Chloride; (9) Cobalt Chloride; (10) Zinc Chloride; (11) Copper Chloride; (12) Chromium Trioxide

*A = Gram Positive

B = Gram Negative

parameters such as the volume of toxicant and inoculum concentration the method was found to yield reproducible results when metal compounds were substituted for antibiotics. By preparation of diffusion gradient graphs it was possible to determine MIC levels of metal compounds. The diffusion gradients indicate that metal compounds generally diffuse through agar at an exponentially decreasing rate and that this rate is metal specific. Thus it is not possible to relate zone size directly to metal toxicity in comparative tests for different metals. However, when only one metal compound is being tested on a number of bacterial species, the zone size can be used to give a measure of comparative toxicities.

Where zone of inhibition of bacterial growth is small, a more accurate determination of the MIC may be achieved by repeating the experiment using a more concentrated metal solution. In this way it will be possible to measure the MIC in a non-exponential region of the diffusion curve.

The variations between results obtained using Noble agar and Nutrient agar in combination with different inoculum strengths demonstrates the importance of quantifying MIC values accurately (Fig. 2.17). In addition, if the mechanisms of the compound diffusion are not understood, incorrect conclusions could be drawn from the MIC values determined. For example, the apparent absence of an effect by lead on certain bacteria (Marques et al 1979; Nakahara et al 1977) may not be due to the presence of resistant organisms but could be attributed to

the strong binding of lead in the media, restricting its availability to the bacteria. This hypothesis is supported by the results obtained in this study concerning the diffusion of lead using Nutrient agar and Noble agar (Fig. 2.16). Similarily, when assessing the MIC values obtained using the agar dilution method (Simon-Pujol et al 1980) allowance must be made for both varying diffusion of metals through, and binding of metals by, the media. In this technique, the bacteria are surface-plated in broth, so that there is a possibility that some cells develop into the lag phase of growth before they become exposed to the metals.

Using agar diffusion the variaiton of zone size measurements hence MIC values, at different phases of growth was clearly demonstrated (Table 2.2). Therefore caution should be exercised when interpretating results of tests to determine bacterial resistance.

The results of viable counts of soil bacteria in metal amended media were used by Duxbury (1981) to determine the concentrations of metals to which bacteria could be considered tolerant. He achieved this by extrapolation of the results using a standard formula. Similarily, MIC readings obtained by agar diffusion could possibly be correlated to initial concentrations in the discs. Relationships between the results of agar diffusion, agar dilution and tube dilution tests could then be clarified and a more realistic assessment of toxicity levels achieved.

The zones of inhibition of bacterial growth are clearly visible on agar plates as is the junction between zones of partial and total inhibition. In no case was there a gradual change in bacterial density between zones of partial and total inhibition; the interface was always well defined. It has been suggested that the zones of partial inhibition are caused by the presence of a number of resistant cells within the strain (Thompson & Watling 1984). However, it is also possible that the particular metal compound causes an extension in the lag phase of bacterial growth. Mitra et al (1975) found that cadmium (in ionic form) caused the E. coli lag phase to be extended. Nevertheless, individual discrete colonies within the zones of inhibition are probably resistant cells resulting from either plasmid mediation or selective mutation.

Areas of increased bacterial concentration which sometimes form at the interface of the normal growth and inhibited growth zones may indicate growth stimulation by the study metal at that precise concentration. However, it is more likely that this increased growth is the result of a greater availability of nutrients due to the presence of dead bacteria in the area together with low levels of the growth inhibiting metal compounds.

The MIC is an expression of bacteriostatic action. In order to assess the bactericidal effect of the study compound it would be necessary to transfer non-colony forming units within the zones of inhibition of bacterial growth to new agar

plates. This can be achieved by using a pre-sterilized velvet stamp pad of the same dimensions as the petri-dish. The pad is pressed onto the surface of the study plate and then re-applied to the surface of a freshly prepared agar plate. After incubation, those bacteria which had not been killed but had merely had their growth retarded would become visible as individual colonies. These colonies could then be used in further bioaccumulation or biotransformation experiments as they may represent resistant cells.

For the purpose of this study, diffusion gradients have been prepared for all study compounds. Under routine conditions, however, it would only be necessary to determine the metal concentration in the agar at a point adjacent to the zone of inhibition of bacterial growth in order to obtain an MIC. Values of metal concentrations causing both total and partial inhibition can be obtained in this way. The technique can therefore be used as a fast, sensitive test to assess metal compound toxicity. Furthermore, bacterial cells from partial inhibition zones can be subcultured so that investigations can be made into the mechanisms of bacterial resistance to metal compounds.

Statistical analysis of the 43 sediment heterotrophic bacteria showed that the Gram positive bacteria were more susceptible to heavy metal compounds than the Gram negative bacteria.

The findings of this study indicated that, of all the elements tested, only the compounds of mercury gave results that were consistently imprecise. This is probably entirely due to the relative inaccuracy that is inherent in the analytical technique for this metal, rather than to any physiological aspects.

2.5 SUMMARY

The agar diffusion technique has been used to determine the comparative bacterial sensitivity and MIC levels to selected compounds of zinc, arsenic, manganese, nickel, chromium, mercury, cadmium, copper, selenium, lead and cobalt. Diffusion gradient graphs prepared for each metal compound define the concentrations of metal available to bacteria at all points across the inoculated agar test plates. Variations in bacterial growth zone types demonstrate the different effects of individual metal compounds on bacterial growth phases. The concentrations of metal compound causing these effects can be quantified using the diffusion gradient graphs.

Analysis of the graphs demonstrate clear differences in diffusion of metal compounds through nutrient agar and indicate that, in the case of lead, there is virtually no diffusion. This lack of diffusion could have led to many organisms being erroneously classified as lead resistant. The use of Noble agar as support media enables lead diffusion experiments to be carried out as lead diffuses rapidly through this material.

The rank order of toxicity of the metal compounds tested to *E. coli* was mercuric chloride>methylmercuric chloride>cadmium chloride>selenium dioxide>zinc chloride>lead chloride>cobalt chloride>nickel chloride>sodium arsenate>chromium trioxide> copper chloride>manganese chloride. The metal compounds were generally more toxic to Gram positive sediment bacteria than to Gram negative strains.

CHAPTER 3

3.1 INTRODUCTION

As a result of man's activities a wide variety of pollutants are constantly being discharged into the marine environment. The types of pollutants involved are as varied as their sources and include oils, pesticides, detergents and metals. Some of the pollutants pose a greater threat to man than others and even within pollutant groups there are variations in relative toxicity of individual compounds.

Amongst the metals, mercury, cadmium and their compounds are the most toxic. Chronic cadmium poisioning results in rheumatic disease. The outbreaks of itai-itai disease in Japan have been directly linked to contamination of the environment with cadmium (Kobayashi 1971). Mercury poisoning, recognised as early as 1953, gives rise to the clinical condition of Minamata disease. Although this is classically linked with the consumption of contaminated fish and shellfish, a great number of deaths have resulted from the consumption of mercury-treated seed (Bakir 1973).

Bacteria are also affected by metal input into the environment and because of their fundamental role in primary production, decomposition and nutrient recycling, any effect on their ecology becomes of major importanct.

Environmental factors and/or bacterial interaction can

alter both the form and the concentration of metal compounds.

The toxicity of metals to micro-organisms can be reduced by binding the metal to organic materials in the environment, e.g. humic acids, clays, proteinaceous materials. Similarily reduction of metal activity occurs in the presence of chelating agents (citrate, cysteine) many of which are used in laboratory media. Gadd & Griffiths (1978) discuss the reduction in metal toxicity, that occurs when complexing agents are present, either in the environment or laboratory media.

Interactions with other metals compounds or with magnesium and calcium cations may also reduce toxicity of metals to bacteria. Conversely a lowering of pH can render the metal ions more available to the biota. The chemical form of the metal is also an important factor in toxicity e.g. dimethyl mercury and tetramethyl lead are both more toxic than the chlorides of these metals.

Bacteria exhibit different mechanisms of detoxification. Those bacteria that produce hydrogen sulphide as a result of their metabolic processes often exhibit tolerance to metals. This is due to the formation of insoluble sulphides. As a result of this the sulphide producing organisms can protect more sensitive species (Gadd & Griffiths 1978).

Many metals in low concentrations are essential growth

factors for bacteria and consequently bacteria have inherent mechanisms for metal uptake. Some bacteria, however, are able to accumulate high concentrations of metals and thereby also detoxify their surrounding environment. Metal uptake may be non-specific and simply involve binding onto cell surfaces, slime layers, capsules, etc. Such mechanisms have both ecological and commercial significance in that removal and recovery of metals can be achieved in this way (Remacle & Houba 1983). An alternative method of metal uptake involves metabolism-dependent transport. This often involves a greater amount of uptake than is effected by simple binding systems.

An alternative method of microbial resistance to metals is that of biotransformation. By this process the metal can undergo a change in valency state and/or conversion to an organometallic compound. Both processes are a form of detoxification of the metal by the bacteria.

In some cases of metal resistance by bacteria, the mechanism seems to be one of decreased uptake, and this may be due to structural differences in cell walls. Babich & Stotzky (1978) reported the greater tolerance to metals of Gram negative bacteria than Gram positive. The present study found similar trends for several metal compounds tested (Chapter 2). However, this effect may be due to specific efflux systems (Foster 1983).

Bacterial resistance to metals and the plasmid determined linkage with antibiotic resistance is well documented (Mietz & Sjogren 1982; Devanas *et al* 1980; Marques *et al* 1979).

The effect of both sewage and metal input into the environment may well pose a serious health hazard. Therefore there is an urgent need for studies which measure the possible co-selection for antibiotic resistance due to selective pressures of metals.

Nakahara *et al* (1977) found 92% of *E. coli* and 96% of *Ps. aeruginosa* isolates from inpatients in Japanese hospitals to be resistant to 400 µg/ml $CdCl_2$ while 57% and 75% (respectively) were resistant to 10 µg/ml $HgCl_2$.

Sjogren & Port (1980) reported that 96% of bacterial isolates from a lake were resistant to one or more heavy metals. Mixed populations of soil bacteria isolated from a mining site were used by Olson & Thornton (1981) to assess relative toxicity of cadmium, zinc and lead. The results indicated that lead was the least bactericidal of the three metals.

The aim of this study was to investigate metal/bacteria interactions with specific reference to Eastern Cape sediments. One advantage of studies in this area is the presence of adjacent sites which differ markedly in the degree of metal pollution.

Therefore it was possible to determine if there was any relationship between background levels and MIC's of selected metals to sediment bacteria.

The choice of E. coli as the test organism was based on the knowledge that there was sewage input at several of the study sites. In addition, MIC readings could easily be obtained by reference to standard graphs (Chapter 2) and any variability in these MIC levels, due to the titration effect of bioaccumulated metals, would be minimised. Also laboratory data was available to effect comparisons.

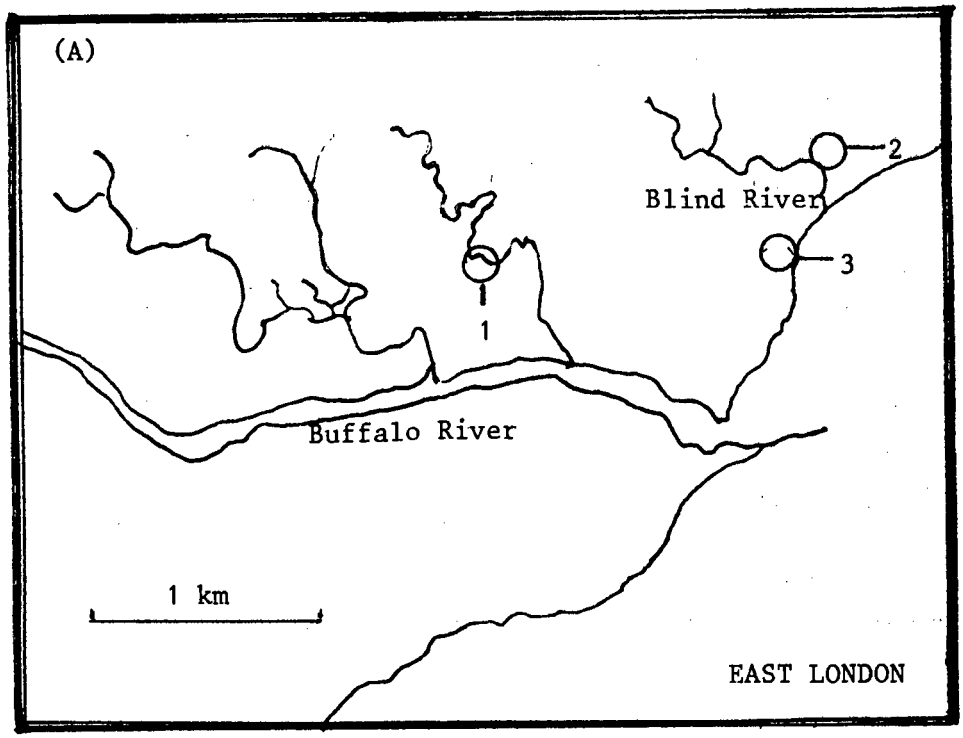
3.2 MATERIALS AND METHODS

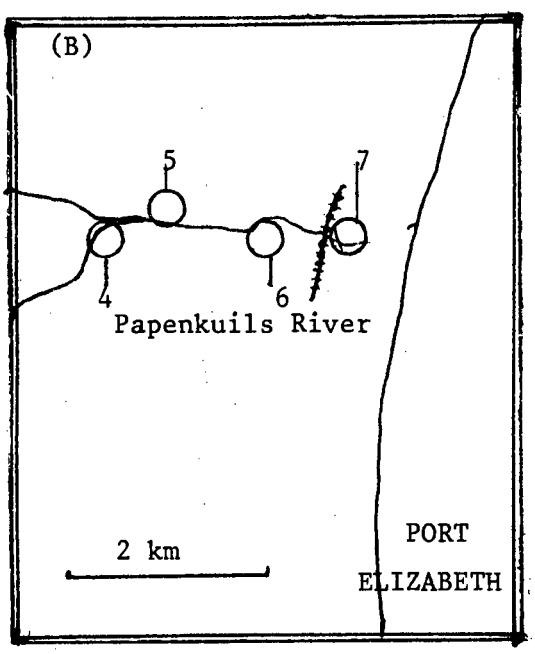
3.2.1 Media

All media used are listed in the Appendix.

3.2.2 Collection of samples

Samples were collected with a sterile PVC corer (Chapter 2) and transferred immediately to sterile bottles. The sample sites were chosen on the basis of results from previous surveys (Watling & Watling 1982b; Watling & Emmerson 1981; Watling et al (pers. comm.) to present metal-polluted (Fig.3.1) and unpolluted areas. The samples were collected at the edge of each river during a low tide cycle.





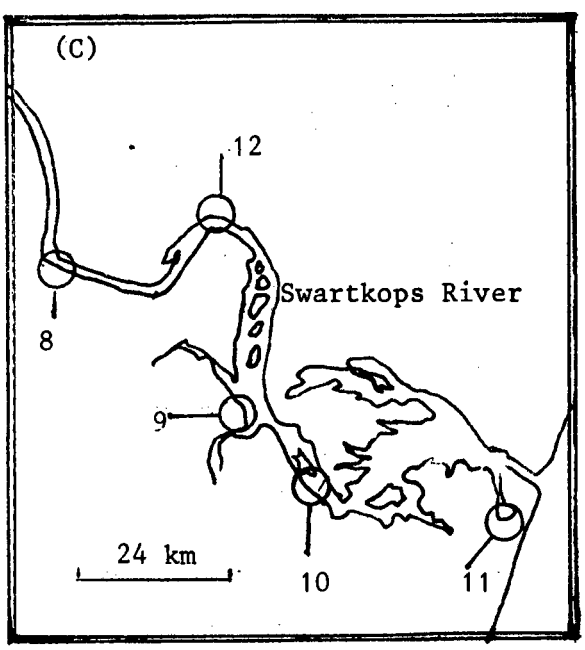


Fig. 3.1 Location of Sample Sites
(A) Buffalo & Blind Rivers, East London
(B) Papenkuils River, Port Elizabeth
(C) Swartkops River, Port Elizabeth

3.2.3 Analysis of sediment for metal levels

The sediments were digested and then analysed as detailed in Chapter 2.

3.2.4 Isolation of bacteria

Aliquots of approximately 2,5 g of sediment were added to 100 ml each of MacConkey purple broth and 1% brilliant green bile broth. After 18 h incubation at 37°C subcultures were plated onto MacConkey purple agar. These subcultures were incubated at 44,5°C (± 0,5°C) for 18 h. In order to isolate axenic cultures of E. coli type I, the following tests were performed on at least ten individual yellow coloured colonies from each culture per site -

- indole production; using an 18 h culture at 44,5°C and Ehrlich's indole reagent (Appendix A);
- citrate utilisation; as shown by growth in Simmons citrate agar at 44,5°C after 18 h incubation;
- lactose fermentation; as shown by acidity and gas in MacConkey purple broth after 18 h incubation at 44,5°C.

Five cultures from each site (with the exception of site 5 where only four cultures were available) were maintained in axenic culture on tryptone soya agar. The cultures were stored at 20°C in the dark for a period of no longer than one week, prior to testing, for metal and antibiotic susceptibility.

3.2.5 Antibiotic susceptibility test

The standardised single disc method of Bauer et al (1965) was used to determine antibiotic susceptibility.

The antibiotics used and relevant concentration are listed in Table 3.1.

The discs were cut from ordinary commercial blotting paper which was first tested to ensure it contained no toxic elements. The antibiotic solutions were added to the discs with an Eppendorf pipette ½ h prior to the test.

3.2.6 Metal susceptibility test

The modified agar diffusion test was used. All materials and method for this test are detailed in Chapter 2. The metal compounds chosen for testing the environmental bacterial strains were the chlorides of copper, cobalt, zinc, nickel, cadmium, mercury and lead and the trioxide of chromium. This choice of metals was determined on the basis of results of previous analyses of the sampling sites. Manganese was excluded as it was considered to be relatively non-toxic to Gram negative heterotrophs (Chapter 2).

3.2.7 Statistical analysis

An MIC level for each of the 59 isolates for each

TABLE 3.1

Antibiotic solutions

Antibiotic	Source	Final. Conc. (µg)
Tetracycline	Reverin 1M 350 mg	30
Kanamycin	Kanamycin Nova injection Novo Industries	30
Bactrim	Stock (1M) Co-trimoxazole: Roche	320
Chloram-phenicol	Chloromycetin succinate: Parke Davis	30
Gentamycin	Garamycin (1M): Scherag	40
Ampicillin	Penbritin Stock 1V: Beecham Research Labs.	10

of the eight metal compounds was calculated. The exponential fit formula -

$$y = a.e^{bx}$$

was used in the case of cadmium, copper, nickel, zinc, cobalt and chromium.

The formula -

$$y = a.x^{b}$$

was used for lead and mercury. These formulae were chosen on the basis of the results of diffusion gradients (Chapter 2).

Concentrations (µg/g) of the eight metal compounds were compared to MIC levels (µg/g) of the environmental isolates to determine if there was any correlation between the two. The statistical technique applied to the data was that of least squares analysis.

The mean MIC for each metal at each site as well as the mean of the total MIC determinations (all sites) for each metal was compared to the MIC levels of the stock culture (NCTC 10418). Relative

susceptibilities of environmental and laboratory isolates to each metal were thereby assessed.

3.3 RESULTS

3.3.1 Analysis of sediments for metal concentrations

The levels of metal concentrations at each site are shown in Table 3.2 The average "high" concentration levels of metals for the eastern Cape area are 30 µg/g for chromium; 50 µg/g for zinc; 20 µg/g for copper and nickel; 0,1 µg/g for cadmium and mercury; 10 µg/g for cobalt and 40 µg/g for lead.

A comparison of the two sets of data demonstrated that both metal-polluted and unpolluted areas were included in the study. Each metal compound was present at a range of concentrations and these were site specific.

3.3.2 Antibiotic susceptibility

The zones of inhibition of growth for each of the 59 strains and a control strain (NCTC 10418) were recorded. The results were then interpretated according to Bauer *et al* (1965) and SAIMR, Port Elizabeth, as sensitive, intermediate and resistant for each antibiotic tested. The results are listed in Table 3.3. All strains were resistant to chloramphenicol including the control and so these results were excluded from subsequent analysis.

TABLE 3.2

Metal concentrations in sediment samples

(concentration µg/g)

Site	Cr	Zn	Cu	Ni	Cd	Hg	Co	Pb
1	100,4	111,1	1,0	5,0	<0,01	0,002	<0,1	60,2
2	24,0	145,5	2,5	3,4	0,53	0,021	<0,1	84,6
3	5,5	4,3	1,2	<0,1	<0,01	0,010	<0,1	<0,1
4	8,2	32,3	3,3	1,2	0,04	0,160	0,4	36,0
5	71,4	247,6	21,4	5,2	0,40	0,390	1,9	67,6
6	87,4	381,0	52,4	11,7	0,48	0,410	2,9	99,0
7	6,5	15,5	0,9	0,5	0,05	0,070	0,5	1,4
8	10,8	16,2	4,1	14,4	<0,05	0,009	2,2	5,4
9	16,1	76,6	12,1	18,9	<0,05	0,010	2,4	79,0
10	22,5	130,0	3,0	23,5	0,05	0,012	2,0	129,0
11	18,1	14,2	3,9	1,5	0,05	0,017	0,5	4,9
12	19,4	28,7	7,9	8,3	<0,05	0,010	4,2	8,8

TABLE 3.3

Antibiotic susceptibility
of environmental isolates of E. coli

SITE	30 µg Tetracycline			30 µg Kanamycin			320 µg Bactrim			30 µg Chloramphenicol			40 µg Gentamycin			10 µg Ampicillin		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
1	1	3	1	0	0	5	0	0	5	5	0	0	0	0	5	1	0	4
2	0	5	0	0	0	5	0	0	5	5	0	0	0	0	5	0	0	5
3	1	1	3	0	0	5	0	0	5	5	0	0	0	0	5	0	0	5
4	5	0	0	0	0	5	0	2	3	5	0	0	0	0	5	0	2	3
5	5	0	0	0	0	5	0	0	5	5	0	0	0	0	5	5	0	0
6	5	0	0	0	3	2	0	0	5	5	0	0	3	0	2	0	0	5
7	5	0	0	0	1	4	0	1	4	5	0	0	0	3	2	0	1	4
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	5	0	0	0	0	5	0	4	1	5	0	0	0	0	5	2	0	3
10	5	0	0	0	0	5	0	5	0	5	0	0	0	0	5	3	0	2
11	2	1	2	0	0	5	0	0	5	5	0	0	0	0	5	0	0	5
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

R : Resistant

I : Intermediate

S : Sensitive

0-5 : Number of bacterial strains

3.3.3 Susceptibility of E. coli to metals

The means of the zone size measurements are shown in Table 3.4. The MIC of each metal to each strain was calculated from the individual zone size measurement by reference to the diffusion gradient graphs. The means of these values for each site were calculated, and with the exception of lead, were all within a limited range (Table 3.5). The MIC's of the environmental isolates correlated well when compared to those of the laboratory stock culture. The exception was lead, where several very high MIC values were found to environmental bacteria and there was a marked variation between the highest and lowest MIC value found for most of the isolates within each site (Table 3.5).

3.3.4 Statistical analysis

Statistical analysis (comparison of means) showed that with two exceptions there was no correlation between background levels of metals and high MIC values to environmental bacteria ($p>0,05$ that the hypothesis is correct). The two exceptions were for cobalt where there was a positive correlation of the data ($p = 0,0009$) and for chrome where an inverse relationship was found ($p = 0,002$). Scattergrams prepared from the results of these analyses are shown in Figures 3,2 to 3,5. It was not possible to determine a statistical comparison

TABLE 3.4

Comparison of zone size measurements of *E. coli* from the environment

Site	Mean ($\Sigma_i^n X_i/n-1$) zone size (mm)							
	Cr	Zn	Cu	Ni	Cd	Hg	Co	Pb
1	8,5	7,0	3,6	2,6	6,4	8,4	7,1	1,5
2	6,9	6,9	0,7	3,9	5,9	10,7	8,1	2,1
3	6,9	7,3	0,7	2,9	7,1	10,3	8,3	1,7
4	6,3	6,6	1,7	2,6	6,5	9,8	7,0	7,0
5	6,3	6,1	2,0	2,9	6,5	10,8	6,5	7,6
6	6,8	6,2	2,8	4,0	7,6	10,7	6,8	9,7
7	6,3	6,2	1,6	3,7	6,8	10,3	7,0	6,6
8	4,8	5,2	2,1	3,1	6,0	9,4	6,2	3,0
9	5,3	5,9	2,7	2,9	5,6	9,8	6,4	5,9
10	6,1	6,2	2,7	3,7	6,8	7,1	6,8	5,8
11	6,1	6,2	3,9	3,8	6,9	10,8	6,8	4,2
12	6,0	4,2	3,3	4,3	6,0	9,9	8,2	5,5

TABLE 3.5

Comparison of means and ranges of MIC's of metals to environmental and laboratory E. coli

Site	Cr		Zn		Cu		Ni		Cd		Hg		Co		Pb	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	127	35	21	6	98	37	89	59	45	19	16	2	50	13	298	359
2	136	17	21	3	131	26	70	32	48	10	11	3	42	2	173	324
3	144	15		6	132	21	84	81	40	8	12	13	42	24	223	343
4	180	105	22	8	121	55	91	54	44	3	13	8	50	15	17	18
5	154	15	25	6	118	45	85	41	46	9	12	2	57	8	17	14
6	146	30	23	4	107	53	67	26	37	15	11	3	51	5	24	92
7	152	34	23	8	122	48	78	16	42	16	11	3	51	28	23	27
8	170	25	27	10	112	34	82	10	48	22	13	2	57	28	190	384
9	164	22	24	2	114	50	72	22	52	15	12	1	55	21	42	128
10	154	19	23	6	95	8	69	9	42	23	21	21	52	19	30	61
11	153	19	23	2	103	70	66	56	41	13	11	2	51	16	82	106
12	154	19	33	32	116	51	71	31	46	5	12	2	57	18	30	53
Total 1-12	152	138	24	40	114	76	77	99	44	30	13	22	51	42	97	395
LAB.	127	0	30	6	99	6	65	10	29	4	18	3	46	10	35	10

1 = Mean

2 = Range

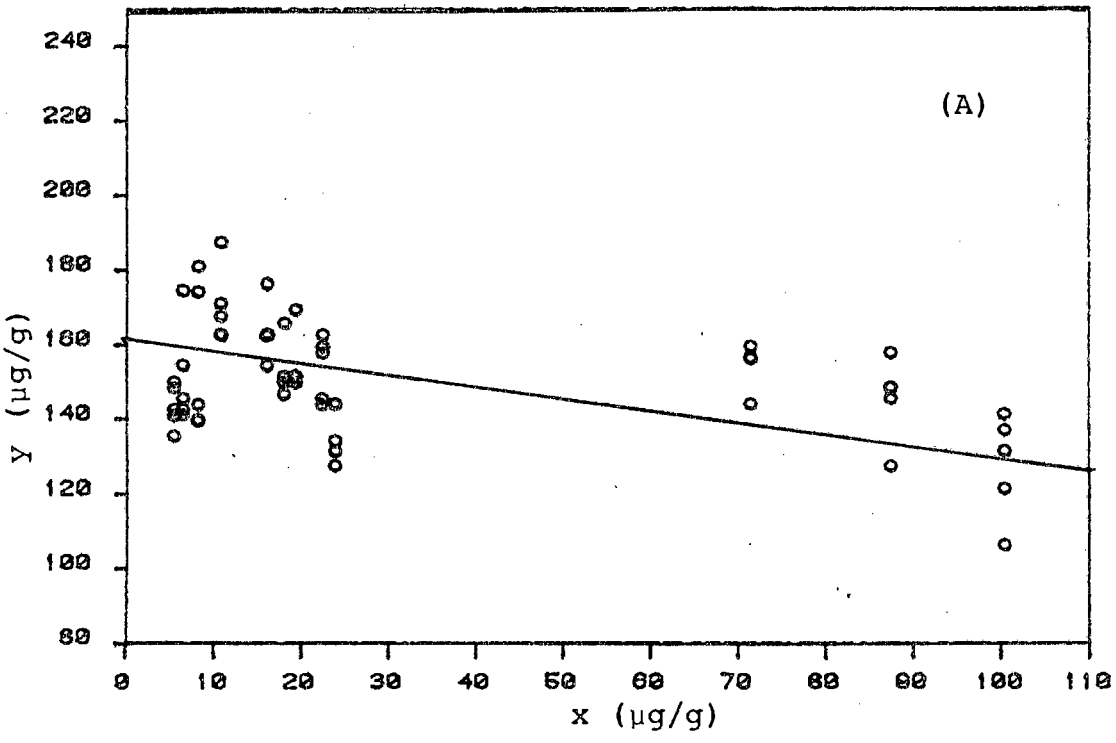
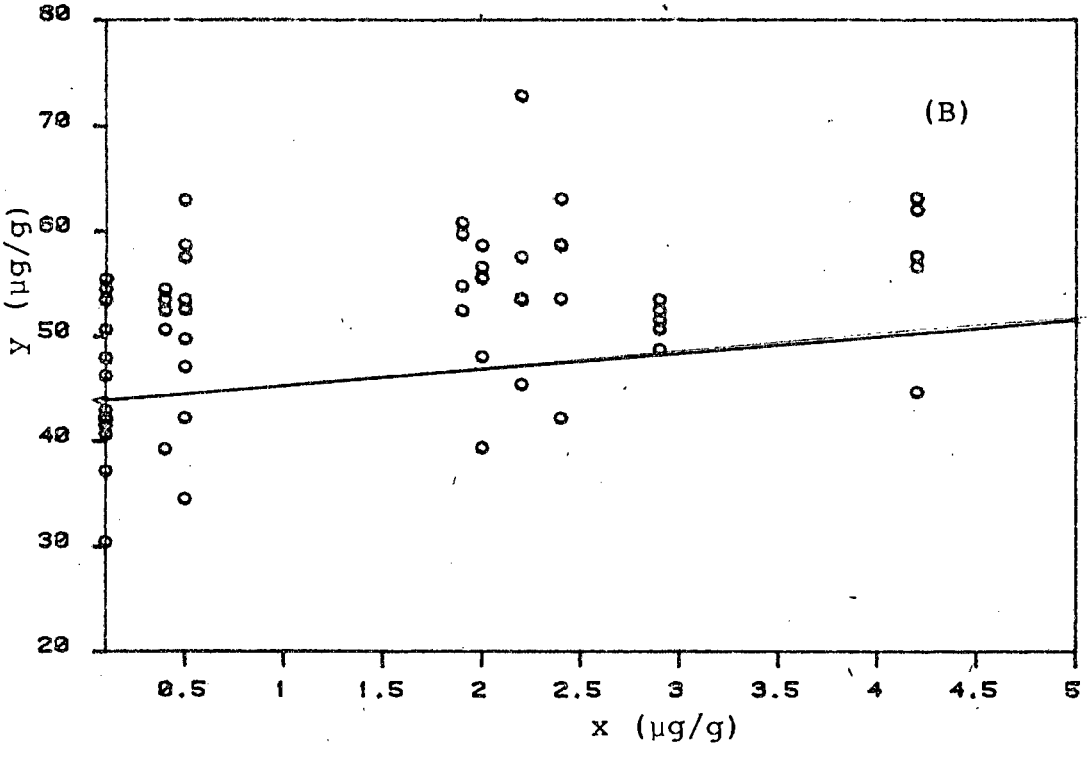


Fig. 3.2 MIC's of environmental isolates (y) versus site sediment concentration (x)

(A) Chrome

(B) Cobalt



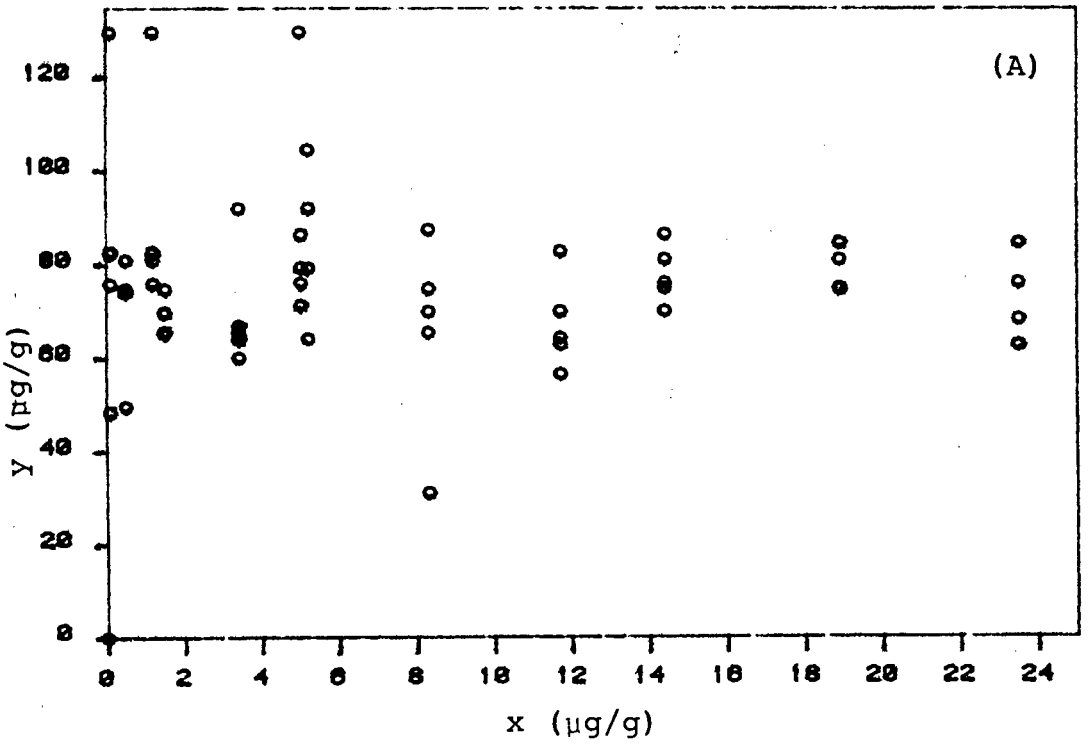
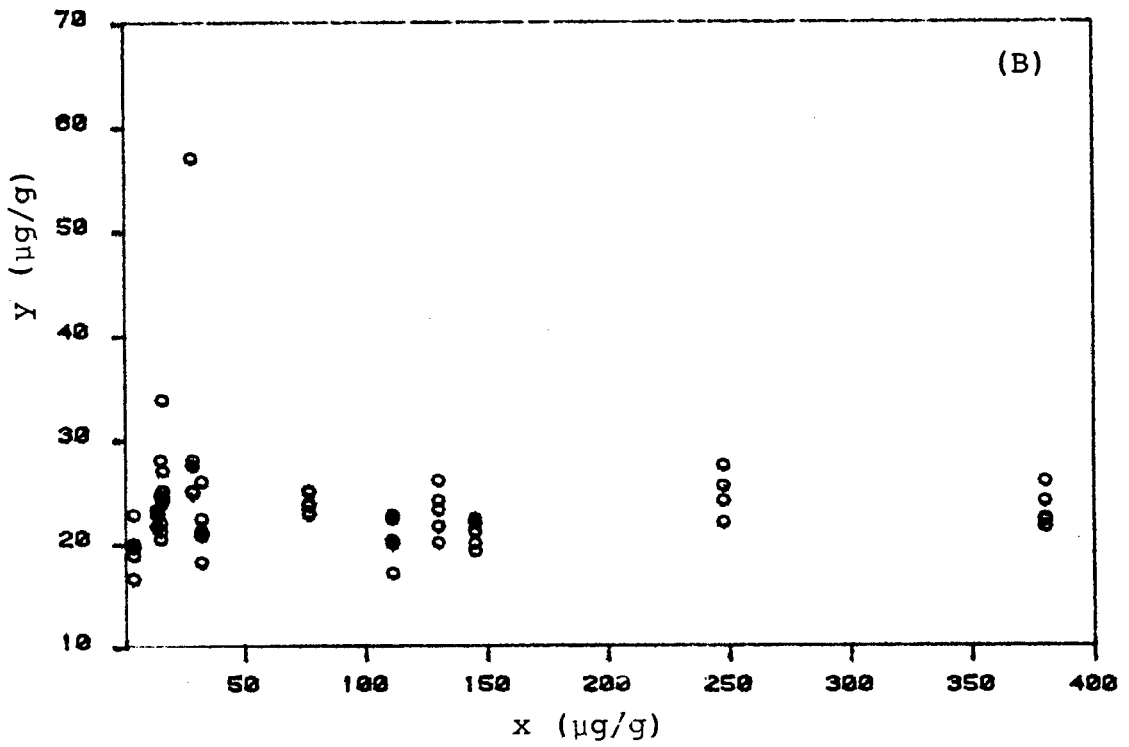


Fig. 3.3 MIC's of environmental isolates (y) vs. site sediment
(A) Nickel
(B) Zinc



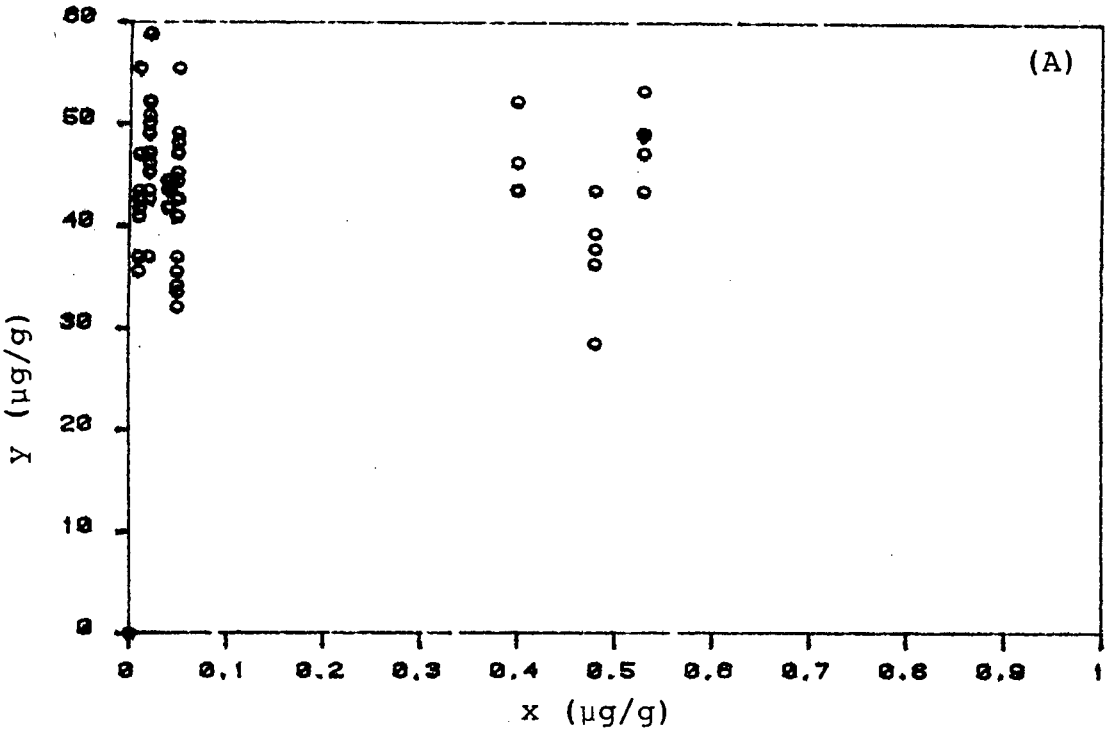
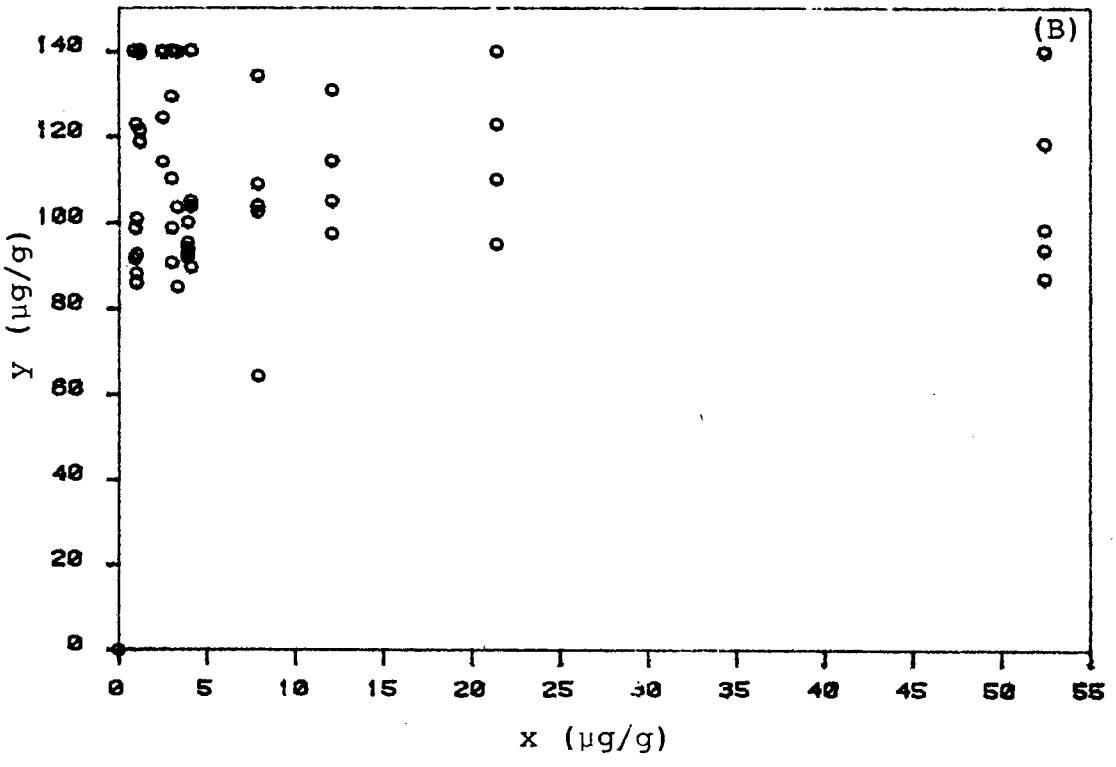


Fig. 3.5 MIC's of environmental isolates (y) vs. site sediment concentration (x)
(A) Cadmium
(B) Copper



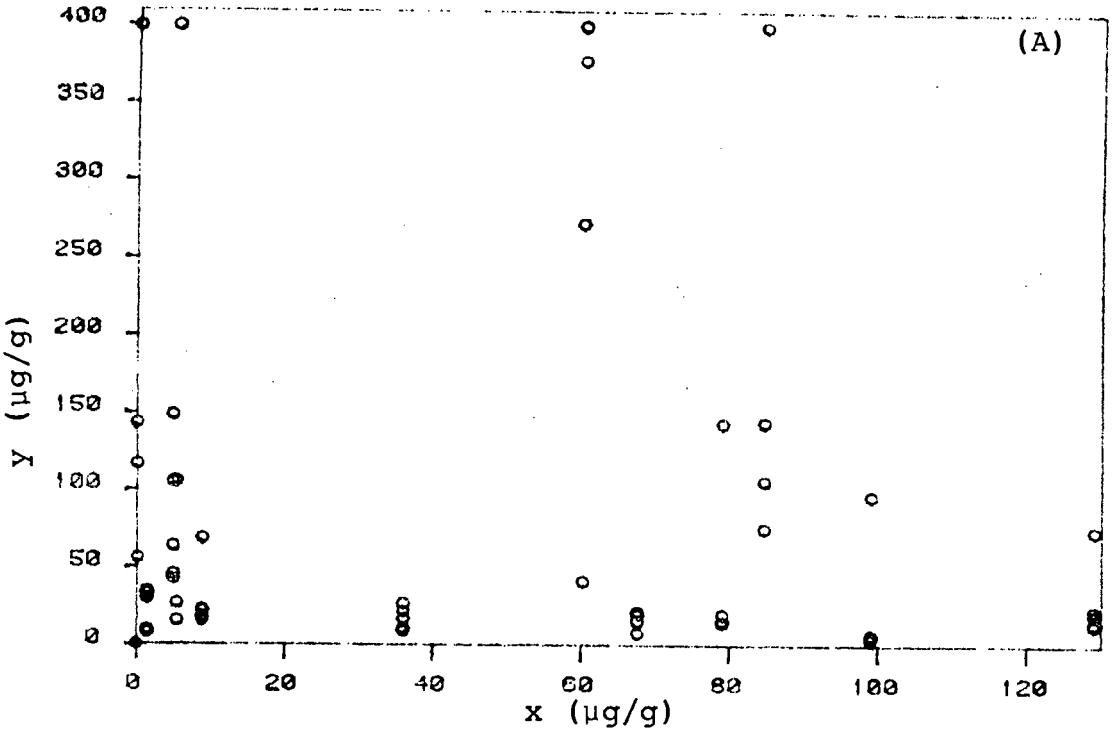
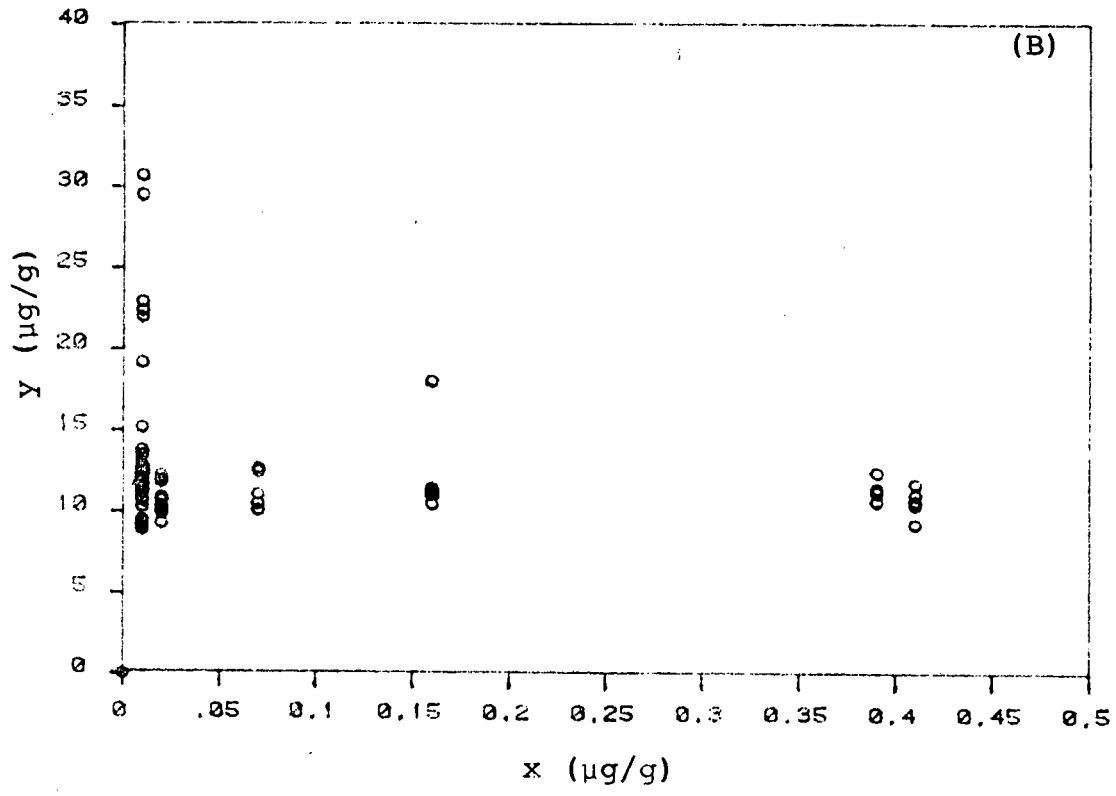


Fig. 3.4 MIC's of environmental isolates (y) vs. site sediment concentration (x)

(A) Lead

(B) Mercury



of samples between field and laboratory data since only one strain of E. coli was available for the laboratory tests. However, a list of mean MIC values at each site for each metal together with the value for the range gives a good indication of any differences. A minimum of a two-fold increase in concentration is generally used in toxicity testing and these results should be viewed accordingly. In this respect the only real differences between field and laboratory MIC levels are found in the results for lead (Table 3.5).

3.4 DISCUSSION

Fifty-nine isolates of bacteria from selected sediments in the eastern Cape were tested for susceptibility to metal compounds and antibiotics. The modified agar diffusion method was used to test bacterial susceptibility to copper, lead, nickel, zinc, chromium, mercury, cobalt and cadmium. Single disc diffusion was used to test for susceptibility to tetracycline, chloramphenicol, gentamycin, kanamycin, bactrim and ampicillin. The selection of metals to be used in the experiments was based on previous metal analysis of sediments from the study sites. Subsequent analysis of the sediments collected for this study showed that, with the exception of cobalt, there was a wide range of concentrations for each metal under test. Cobalt concentrations were low

throughout the entire study area. The results of the metal and antibiotic susceptibility tests indicated that there was no evidence of multi-metal and/or multi-antibiotic resistant E. coli in the eastern Cape despite the presence of increased metal levels in some sediments.

With the exception of cobalt, no positive correlation was found between increased environmental metal levels and high MIC values to those metals. It must be remembered that the form of the metal in the environment may be considerably different from that under test conditions and therefore account for this lack of correlation. The cobalt correlation was interesting as sediment analysis indicated lack of high concentrations of cobalt in all samples tested. It was felt that this result may be an anomaly since the differences between sediments levels were small as were the differences between MIC values. Similarily, the inverse relationship between chromium trioxide and MIC needs further investigation. One explanation for this phenomena is that the form of chromium may be changed on entering the environment and absorption and/or adsorption onto sediment surfaces together with co-precipitation as hydrated iron oxides, may render it less toxic to bacteria. It was noted that there were always individual specks of matter adjacent within the zone of inhibition produced by chromium trioxide. This could either be the result of resistant colonies or some form of metal/media interaction. Such an interaction was shown when CrO_3 was added to nutrient broth in tubes (Chapter 4). It is therefore postulated that the inverse

correlation between chrome and MIC could be due to a change in the metal form and that this change is more pronounced at higher concentrations. Chrome was found (Chapter 2) to exhibit a greater toxicity to Gram negative organisms than to Gram positive. The possibility that an alteration in metal form may alter its molecule size and therefore diffusibility through bacterial cell walls must be considered. The results of metal susceptibility tests indicated there were no multi-metal resistant bacteria, similarily there was no evidence of multi-antibiotic resistance from the same samples.

The results of the antibiotic susceptibility tests indicated that there was no relationship between heavy metal levels in the environmental samples and antibiotic resistance. These results were not subject to statistical analysis as the conclusions could be drawn by visual comparisons of the data. The range of antibiotics tested was limited but did include those normally used in treatment of Gram negative infections. The general trend was that the bacteria exhibited resistance to chloramphenicol and tetracycline only. It was felt that the chloramphenical results should be disregarded in view of the finding that the known sensitive control culture was also resistant to this antibiotic. As a 'resistant' control an environmental strain of Pseudomonas was tested to the same set of antibiotics. This control proved sensitive only to gentamycin.

The results of these assays indicate that in the Eastern

Cape the levels of the metals found in this study do not act as co-selectors for antibiotic/metal resistance for E. coli.

With the exception of lead, the MIC's of the metals to both the laboratory stock cultures and the sediment are similar. However, since only one laboratory strain was available for comparison further strains should be tested before definite conclusions can be drawn.

The findings of this study do not agree with those of other workers (Nakahara 1977; Seyfried 1980; Marques et al 1979; Simon-Pujol 1980). The high proportion of lead resistant organisms found by these workers may be a reflection of the unavailability of lead to the bacteria under their test conditions rather than due to its lack of toxicity. This unavailability could be caused by binding of the lead to media constituents (Chapter 2).

The agar diffusion method determines the action of toxicants to bacteria in the lag phase of growth. The actual concentration of metals causing toxicity are quantified. As such it is therefore a very sensitive indication of metal toxicity. A formula for the comparison of results of agar dilution to agar diffusion could determine correlation, if any, between the results of the two methods provided standardisation of parameters (media, etc.) was achieved for

the agar dilution method. Therefore there is an urgent need for the standardisation of methodology and for a rationale for interpretation of results in metal toxicity/bacterial testing.

3.5 SUMMARY

The agar diffusion method was used to test antibiotic and metal toxicity to 59 isolates of *E. coli* which were isolated from Eastern Cape sediments. The results indicated that there was little correlation between levels of metal concentrations in the sediment and resistance. Co-selection for antibiotic resistance in metal loaded sediments was not found. Differences between resistance levels of sediment strains of *E. coli* and *E. coli* (NCTC 10418) were found for only one of the eight metal compounds tested.

CHAPTER 4

4.1 INTRODUCTION

Both chemical and biological assay techniques are required in order to assess the environmental impact of effluents and quality criteria for them. Chemical techniques can only be used to determine concentrations of specific known toxicants so that it is possible to miss other toxic substances which could be present. Furthermore, chemical studies alone cannot be used to quantify synergistic or antagonistic effects which may result with mixed effluents. The use of bioindicator organisms is therefore essential to determine the effects of effluents in the environment.

Bacteria satisfy the criteria for indicator organisms (Phillips 1977). Their culture in the laboratory is comparatively simple and test results can be obtained rapidly using a minimum of sophisticated equipment. The use of mixed bacterial cultures as bioindicators has been described by Bauer *et al* (1981) who measured biological oxygen demand (BOD) in a rapid bioassay method. Busch (1983) evaluated this method and stated that "toxicity studies are potentially so complex that a simple procedure using relative responses data from a sensitive repeatable controlled test system using bacteria becomes attractive". However, a disadvantage of the BOD system is that it measures total toxic effect only. Unless tests are repeated with individual components the factors affecting such toxicity remain unexplained.

Toxic elements rarely enter or remain in the environment as single element forms. Industrial wastes often contain several toxicants in each effluent, e.g. battery manufacturing industry (Pb, Cd); paint industry (Pb, Cr) and electroplating industry (Cr, Cd, Cu). When such effluents are combined with other discharges, e.g. sewage (Hg, Ni, Zn, Cu), pesticide run-off (As, Zn, Mn) the potential toxicity to the biota is very high. In marine as well as other systems the disturbances caused by such toxic wastes can exert effects at all trophic levels and subsequently on man.

It is therefore surprising that very little literature is available on synergistic and/or antagonistic effects of metal combinations. However, the research studies that have been undertaken on this subject demonstrate that such effects can occur.

Furmanska(1979) determined the effects of copper, zinc and iron on fish and found that the values of concentration LC_{50} - 48 h exhibited a three-fold decrease upon cumulative action. A combination of mercury, lead and zinc was found to have an increased toxic effect on marine ciliate protozoa compared to the individual elements as measured by growth rate (Gray 1974). Aquatic bacteria demonstrated increased uptake of chromium, cobalt, iron, manganese and zinc when arsenic and mercury were added to the water column in increasing concentrations (Guthrie & Singleton 1977).

To test the effects of metals on soil bacteria, Olson

and Thornton (1981) used zinc, copper and lead as single element forms and in combination. They found that pronounced differences in toxic effects did not occur between two and three metal combinations but that the latter was markedly more toxic than individual elements. Of the three metals tested, cadmium exhibited the highest number of synergistic effects and these effects were lessened at lower concentrations of the metal. It is therefore essential not only to determine the cumulative effects of metals but to determine the concentrations at which these effects occur. In order to do this, the tube dilution and agar diffusion method were used to examine both effluents and stock metal solutions for synergism and/or antagonism of metal compounds. E. coli and B. subtilis were used as test organisms.

4.2 MATERIALS AND METHODS

4.2.1 Stock cultures of bacteria

E. coli NCTC 10418 and B. subtilis SATCC Bac3 (840201) were maintained on tryptone soya agar at 20°C.

4.2.2 Stock metal compounds

The preparation of metal compounds is detailed in Chapter 2. Methyl-mercuric chloride was not included. Mercuric chloride was used initially at 500 µg/ml and subsequently at 50 µg/ml.

4.2.3 Media

All media used are listed in the Appendix.

4.2.4 Tube dilution MIC tests

The stock E. coli culture was incubated in nutrient broth for 18 h at 37°C prior to further dilution in nutrient broth to a final concentration of $\underline{c}$ 1,5 x 10^5 organisms per ml. (The spectrophotometric standard graph method was used to estimate bacterial numbers). Doubling dilutions (range 62,5 to 1000 µg/ml) in sterile distilled water were made of each of eleven stock metal compounds. One millilitre aliquots of each of these dilutions were aseptically added to 1 ml volumes of the inoculated broth culture in sterile tubes.

Incubation was at 37°C for 24 h and 48 h. The lowest concentration, for each metal, to inhibit bacterial growth was recorded as the MIC for that metal. Growth was indicated by the presence of turbidity in the media and confirmed by subcultures on nutrient agar at 37°C for 18 h thereby determining the minimum bactericidal concentration (MCC). Controls of individual metal solutions in broth, as well as inoculated and uninoculated broths without metals, were included.

The tests were carried out in duplicate.

4.2.5 Agar diffusion method

The modified agar diffusion method (Chapter 2) was used to determine zone sizes for each metal compound to the two organisms under test.

4.2.6 Synergism and antagonism

The modified agar diffusion method was used to determine zone size measurements for each metal compound. The tests were then repeated by placing a disc containing the metal under test at the centre of a new plate. Three other metal-impregnated discs were placed at appropriate distances from the central disc. These distances varied according to the zone size measurements of the individual metal compounds.

In this way, after incubation, the outer limit of the zones of each of the metals just touched the outer limit of the central zone. Synergistic and/or antagonistic effects of each of the three metals to the central metal were readily observed. The effects included distortions of zone size and/or shape at the point of contact as well as within the zone areas. Lead chloride was tested on both nutrient agar and 1,2% noble agar media.

4.2.7 Effluent testing

Ten effluents of both industrial and environmental origin were collected and their metal content determined

using atomic absorption spectroscopy.

A 200 ml volume of effluent was evaporated to near dryness in a rotary evaporator. The evaporate was dissolved in a known volume of sterile distilled water. This volume varied with each effluent and was the minimum necessary to achieve a suitable consistency for subsequent pipetting. The effluent was stored at -20°C prior to testing by the modified agar diffusion method. One hundred microlitres of each effluent so prepared was added to the discs in place of metal compounds. In order to obtain maximum zone sizes the tests were performed using 1,2% Noble agar media and 1,2% Biolab agar containing minimal added nutrients and mineral salts.

4.3 RESULTS

4.3.1 Tube dilution MIC tests

The results of the tube dilution MIC's are shown in Table 4.1. A turbidity, unrelated to bacterial growth was found for six out of the ten metals tested. The media/metal controls for these six compounds also showed turbidity, indicating some form of non-bacterial interaction.

From the results obtained for lead and manganese it appeared that these metals were either non-toxic or non reactive. Subsequent tests in two minimal media

TABLE 4.1

Results of tube dilution method to determine MIC's of metal compounds to *E. coli*

Metal Compound		Final Concentration (µg/ml) 500	250	125	62,5	31,25	MIC (µg/ml)	MCC (µg/ml)
$CuCl_2$	(a)	(+)	(+)	(+)	-	-	<31,5	>500
$PbCl_2$	(a)	+	+	+	+	+	> 500	>500
	(b)	+	+	+	+	+	> 500	-
	(c)	(±)	(±)	(±)	(±)	+	62,5	-
$ZnCl_2$	(a)	(+)	(+)	(±)	-	-	<31,5	500
$CdCl_2$	(a)	(+)	(+)	(+)	-	-	<31,5	250
$CoCl_2$	(a)	-	-	-	-	-	<31,5	125
$NiCl_2$	(a)	(+)	-	-	-	-	<31,5	500
CrO_3	(a)	(+)	(+)	-	-	-	<31,5	125
$MnCl_2$	(a)	+	+	+	+	+	> 500	>500
Na_2AsHO_4	(a)	-	-	+	+	+	250	>500
SeO_2	(a)	(+)	-	-	+	+	125	250

(+) = Cloudiness of media resembling bacterial growth

\+ = Growth of bacteria

\- = No visible bacterial growth

(±) = Very faint turbidity

(a) = Tested in nutrient broth

(b) = Tested in 1% tryptone

(c) = Tested in 0,17% tryptone

confirmed the non-reactive nature of lead in certain media and the non-toxic nature of manganese at the concentrations tested (Table 4.1).

From these results, it appeared that the tube dilution method was unsatisfactory for the determination of metal toxicity. Therefore, the synergisitc and/or antagonistic effects of combined metal compounds was tested using the agar diffusion method only.

4.3.2 Determination of zone sizes by agar

The average of the two zone sizes obtained for each metal compound to E. coli and to B. subtilis are listed in Table 4.2. The zone size obtained for 500 µg/ml $HgCl_2$ was considered too large for subsequent experiments testing synergism and antagonism. When this test was repeated at a metal concentration of 50 µg/ml a satisfactory result was obtained.

The results of the zone size measurements for E. coli and B. subtilis indicate the greater susceptibility of B. subtilis to the metals under test. This is in agreement with previous findings (Chapter 2).

All zone sizes were converted to mm distance from the disc in order to determine exact placements for synergism/antagonism experiments.

TABLE 4.2

Zone size measurements[1] (mm) for *E. coli* and *B. subtilis*

	Concentration (100 µg)											Hg (50 µg)	Hg (5 µg)
	Cu	Pb	Zn	Mn	Cr	Co	Ni	Cd	As	Se	Pb[2]		
E. coli	17,7	13,0	24,0	13,0	25,8	23,8	18,8	24,2	33,8	29,6	15,9	38,4	24,0
B. subtilis	27,2	13,0	28,6	13,0	33,2	24,6	20,2	22,8	25,0	36,2	19,3	43,8	25,0

[1]Measurements include 13 mm disc

[2]Tested in 1,2% Noble Agar media

4.3.3 Tests for synergism and antagonism

Each metal was tested against the other ten metals and the results recorded using a two-dimensional matrix (Table 4.3). An increase in zone size of either one or both zones, at the point of contact, was recorded as synergism. Similarily, a decrease in zone size was recorded as antagonism. An alteration of zone type from partial growth to no growth was also indicated as synergism and the reverse of this as antagonism. These variations are clearly indicated in Figures 4,1 and 4.2. The findings were only recorded as such if all four readings for each metal were in agreement. Table 4.4 summarises the results according to which metal compound caused the synergistic or antagonistic effect.

4.3.4 Effluent testing

The sources of the effluents, concentration factor for testing, zone sizes and metal content are shown in Table 4.5. With the exception of samples 3, 8, 9 and 10 which are predominantly mixed urban and industrial effluents, all other effluents tested produced zones of inhibition of bacterial growth. Zones of inhibition of bacterial growth were always produced by effluents which contained increased concentrations of toxic metals. (The relative degree of effluent preconcentration prior to testing must

TABLE 4.3

Synergistic and antagonistic effects of metal compounds to E. coli and B. subtilis

Metal in disc	Cd	Cu	Pb	Zn	Mn	Cr	Co	Ni	Hg	As	Se	Pb*
E. coli												
Cd		A				S						
Cu	A			S		S					A	
Pb												
Zn		S			A	S					A	
Mn				A		S	S					
Cr	S	S		S	S		S					
Co					S	S						
Ni												
Hg												
As			S								A/S	S
Se		A		A						A/S		
Pb*										S		
B. subtilis												
Cd						S				S	S	
Cu									S			
Pb												
Zn								S				
Mn								S				
Cr	S											
Co									S			S
Ni				S	S							
Hg		S					S			S		S
As	S								S		A	
Se	S									A		A
Pb*							S		S		A	

Key : S = synergism

A = antagonism

* = In Noble agar media

Fig. 4.1 Synergistic and antagonistic effects of metals compounds on Escherichia coli

A1 : Determination of zone sizes
Ni (left); Cd (top); Cr (bottom)

A2 : No interaction
Cu (left); Pb (top); Zn (bottom);
Ni (centre)

A3 : Synergism - Cr with Cu; Cu with Hg;
Pb (left); Cr (top); Hg (bottom);
Cu (centre)

A4 : Antagonism - Mn with Zn
Cu (left); Mn (top); Zn (bottom);
Mn (centre)

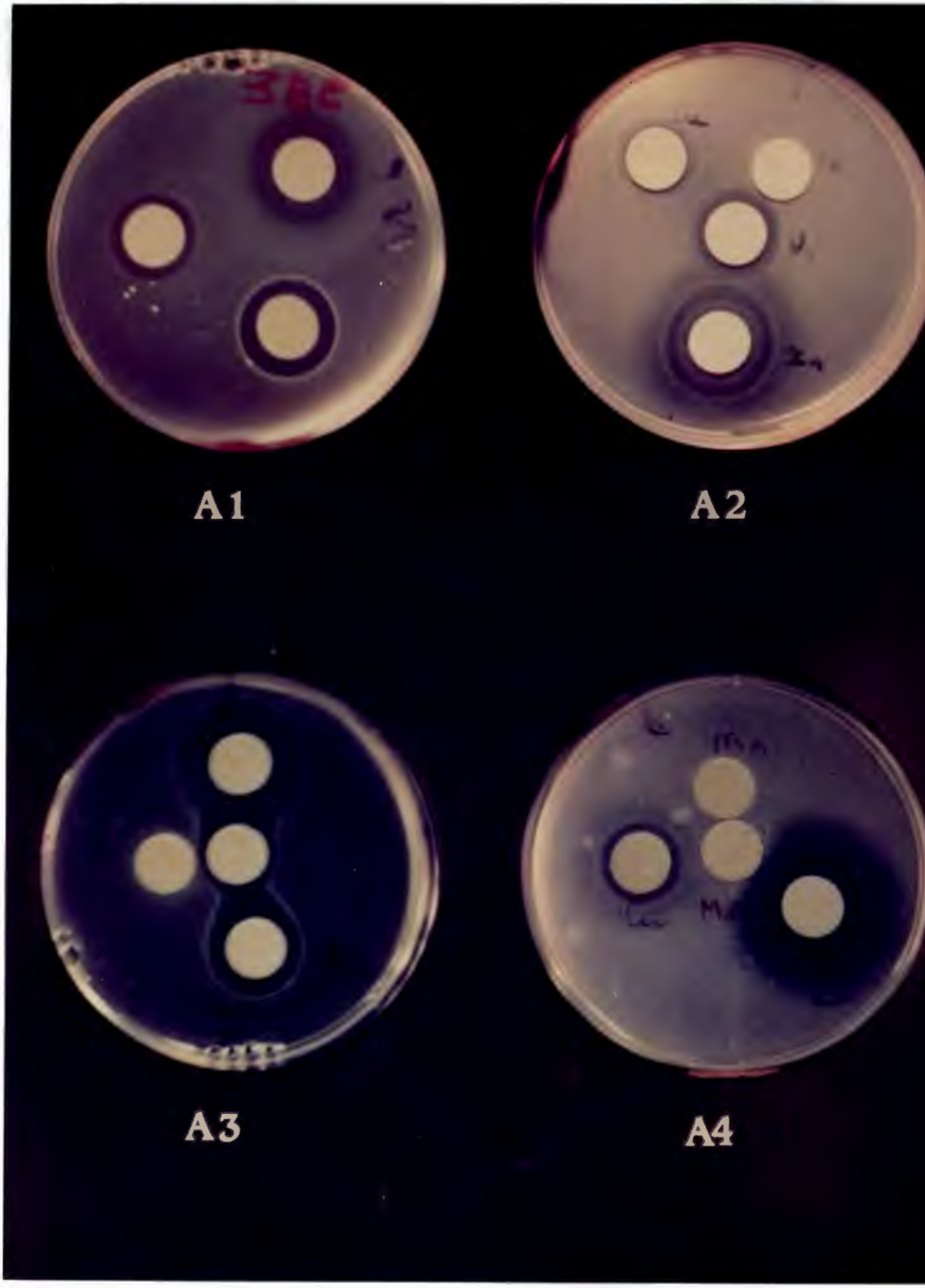

A1
A2
A3
A4

Fig. 4.2 Synergistic and antagonistic effects of metals compounds on Bacillus subtilis

B1 : Determination of zone sizes
Ni (left); Hg (500 µg/ml; top) Cd (bottom)

B2 : No interaction
Mn (left); Cr (top); Co (bottom)

B3 : Synergism - Cd with As
Ni (left); Cd (top); Hg (50 µg/ml; bottom)
As (centre)

B4 : Antagonism & Synergism - As with Se
As (left); Zn (bottom); Se (centre)

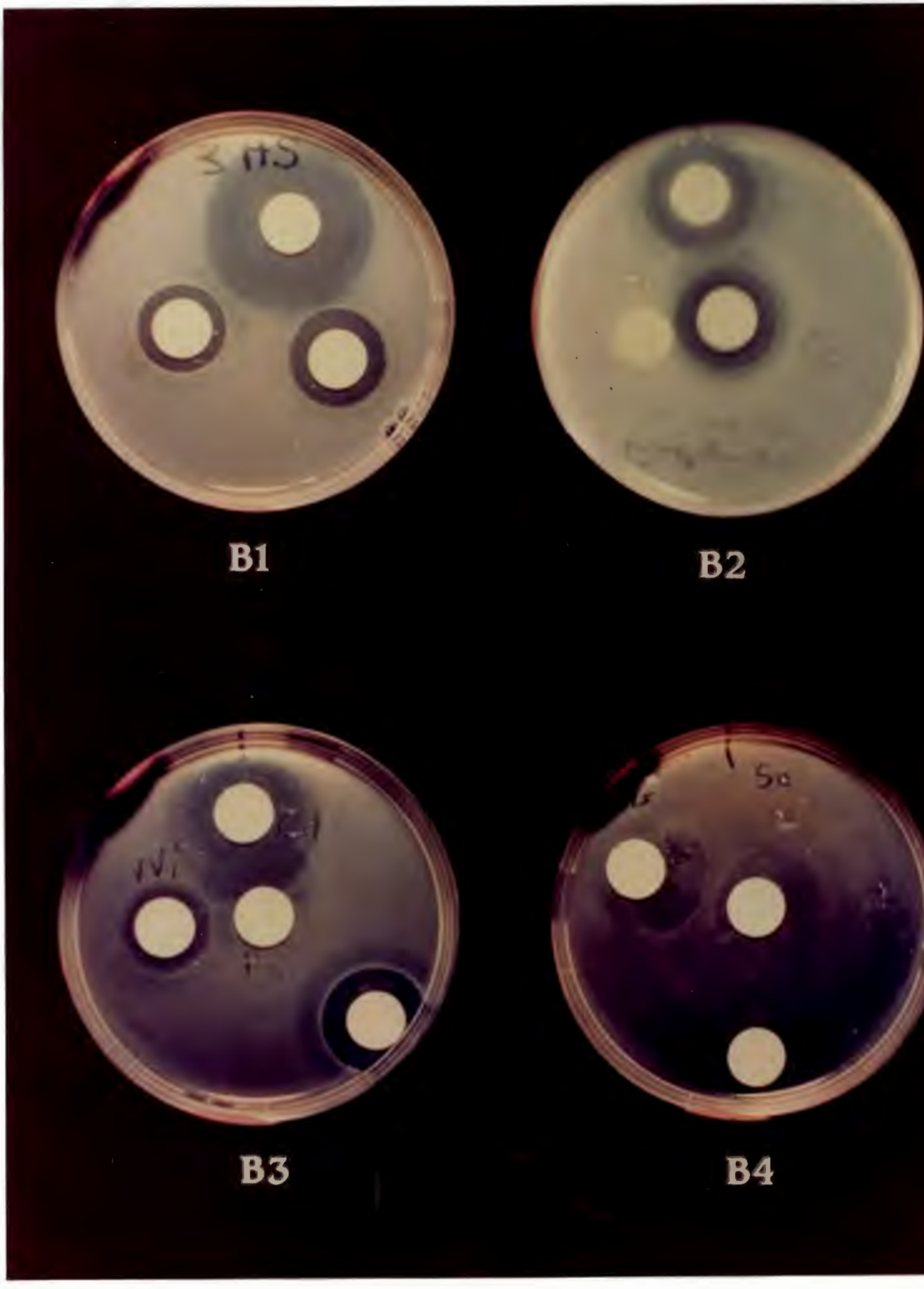

B1
B2
B3
B4

TABLE 4.4

Synergistic and antagonistic effects of metals against selected bacterial species

Organism	Synergism 1	Synergism 2	Antagonism 1	Antagonism 2
E. coli	Cr	Zn	Cu	Se
	Cr	Cu	Mn	Zn
	Cr/Co	Co/Cr	Cu	Cd
	Hg/Cd	Cd/Hg		
	Mn	Co		
	Mn	Cr		
	Cu	Zn		
	Pb	As		
B. subtilis	Cd	As	Pb	Se
	Pb	Hg		
	Hg/Co	Co/Hg		
	Hg	As		
	Mn	Ni		
	Cd	Se*		
	Zn	Ni*		
E. coli & B. subtilis	Cd	Cr	As	Se
	Cu	Hg	Hg	Se
	Se	As	Zn	Se*
	Se	Zn		

*very slight

1 Metal causing the effect
2 Metal on which effect observed

TABLE 4.5

Zone size measurements and metal concentrations of effluents

Sample No.	Conc. Factor	Source of Effluent	Zone Size 1*	Zone Size 2*	Metal concentration µg/l** Cu	Pb	Zn	Fe	Mn	Cd	Cr	Ni	Co
1	40	Wood treatment	45,0	48,0	2100	400	380	700	100	200	1685000	700	6
2	40	Electroplating	23,5	24,0	800	100	630	600	200	70	6000	11700	4
3	13	Paper products	NT	NZ	1700	200	2000	4000	200	10	1000	200	3
4	40	Motor manufacture	16,3	14,7MS	200	200	2700	600	400	6	500	100	4
5	20	Battery manufacture recycled water	23,1	24,2	200	7800	1400	1100	200	6	400	100	4
6	20	Battery manufacture effluent-water	50,0	55,4	200	845000	300	18000	100	10	700	100	6
7	10	Tannery	38,2	40,2	300	200	2800	800	60	50	3720000	100	5
8	40	Sewage	NZ	NZ	100	50	100	600	50	40	300	100	4
9	10	Paper mill	NZ	NZ	100	200	700	4500	700	6	200	50	2
10	10	Sewage	NZ	Nz	300	600	1200	32000	2200	16	100	60	3

NZ = No zone NT = Not tested MS = Moderately sensitive 1* = 1,2% Noble Agar Media 2* = 1,2% Biolab HP Agar media

**After preconcentration

be taken into account when the results for the metal contents of individual effluents are compared with the respective zone sizes obtained under the test conditions).

4.4 DISCUSSION

The use of tube dilution and agar diffusion for the detection of synergism and antagonism of metal compounds was investigated. The results of the tube dilution preliminary tests indicated that several metals were interactive with the media. These interactions resulted either in turbidity of the media (resembling bacterial growth) or reduced toxicity.

Preliminary tests with agar diffusion indicated its suitability as a method for subsequent multi-metal effect experiments.

The results presented in Table 4.4 demonstrate the many synergistic effects that occur when two metal combinations are tested. Although fewer in number, antagonistic effects were also present. By observing which of the two zone sizes showed distortion it was possible to determine which of the metals was creating the effect.

In place of individual elements a solution containing a mixture of elements may be added to the disc. However, the resultant zone size will only indicate comparative synergistic or antagonistic effects. This method therefore has limitations

since it does not determine which of the elements is causing the reaction. In order to further investigate multi-metal effects a series of concentrations of a solution containing lead, chromium, copper, nickel, cobalt, cadmium, zinc, manganese and mercury was added to broth cultures of E. coli in both lag and stationary growth phases. Bactericidal effects were observed at 20 µg/ml for the lag phase cultures and at 30 µg/ml for the stationary phase cultures. These values are lower than most of the individual metal MIC's. The synergistic effect of the solution is thereby confirmed but the elements responsible remain unspecified. Using agar diffusion it is possible not only to determine which metal is exerting the observed effect but also the concentration at which the effect occurs. This can be achieved by reference to standard graphs (Chapter 2) or by removal of agar strips adjacent to the inhibition zone and subsequent determination of metal concentration using atomic absorption spectroscopy.

To study a wider range of metal/metal interactions, additional discs can be prepared using different concentrations of metals or in the case of effluents, different dilutions.

From the results of this study it would appear that synergistic effects of multi-metal compound mixtures occur. The synergistic effects found in this study are in agreement with the findings of other workers (Guthries & Singleton 1977; Furmanska 1979; Anderson & Weber 1975). The antagonistic effects of Cu/Cd and Hg/Se mixtures are in agreement with Watling and Watling (1982a; Förstner (1979) also

discussed the antagonistic effects of Hg/Se mixtures.

An interesting finding was that of the simultaneous occurrence of synergism and antagonism for selenium/arsenic combinations. This may have some significance for the agricultural industry as arsenic compounds are widely used in pesticide preparations.

The results of this study also indicate the applicability of the modified agar diffusion method in effluent testing. Synergistic and/or antagonistic effects of effluents can be quantified and individual effluents monitored for relative toxicity at set time intervals.

4.5 SUMMARY

The use of a bacterial system to test the combined effects of metal compounds and also effluents was investigated. Agar diffusion and tube dilution techniques were tested. Tube dilution was found unsatisfactory due to metal/media interactions. The results of the agar diffusion demonstrated the synergistic and antagonistic effects of metal compounds as well as variations in effluent toxicity.

CHAPTER 5

5.1 INTRODUCTION

The role of bacteria in the alkylation of metals and the mobilization of metals through the environment have been discussed in detail (Jernelov 1975; Förstner 1979).

Metal concentrations in river water are generally higher than those found in coastal or oceanic waters and often vary as a linear function of salinity. The association of the metal as it enters the estuarine environment, may vary between the particulate or dissolved phase and can be altered by several factors including pH and redox potential. The greater proportion of metal input into estuaries reaches the sediments where subsequent interaction with bacteria can play an important role in the mobilization and form of the metal. The transformation of inorganic Hg^{2+} to methyl mercury was first hypothesised by Fujiki in 1963. Subsequently the role played by both aerobic and anaerobic bacteria in the recycling of mercury has been investigated (Pan-hou & Imura 1981; Yamada & Tonomura 1972; Shariat et al 1979). Several workers have demonstrated a correlation between bacterial growth rates and mercury methylation rates (Bisogni 1973; Furatami & Rudd 1980). Bacterial action can result in either conversion of inorganic Hg(Hg^{2+}) to the more toxic methyl mercury form or reduction of Hg^{2+} to Hg° and subsequent volatisation. Similarily sulphide production by inorganic sediment bacteria

can result in the formation of the less toxic HgS compound. The environmental cycling of mercury is shown in Fig. 5.1.

Other important bacterial biotransformations include the formation of methanarsonate and dimethylarsinic acid from arsenate (Braman & Foreback 1973; Wrench & Addison 1981); dimethyltellurite from tellurium compounds and dimethylselenide and trimethylselenonium from selenium (Jernelov 1975; Chau et al 1976). Alkylation of lead by the biota has been considered an abnormal rather than a normal finding (Jarvie et al 1983; Reisinger et al 1981).

Bioaccumulation of metals by bacteria, either directly or as a result of biotransformation, may result in metal compound recycling. Monomethyl mercury formed by bacterial action can be both released into the water column and accumulated at higher trophic levels. Colwell et al (1975) demonstrated that oysters in closed water systems accumulated 200x more mercury when mercury accumulating bacteria were introduced into the systems. There is evidence for the accumulation of copper by bacteria (Baldry & Dean 1980) and also for uptake of cadmium, zinc, lead and copper from simulated wastes (Remacle & Houba 1983). The mechanisms of uptake vary from extracellular binding (Dunn & Bull 1983) to fairly diffuse distribution throughout the cell (Slowick 1981).

Bacteria use both transformation and bioaccumulation as

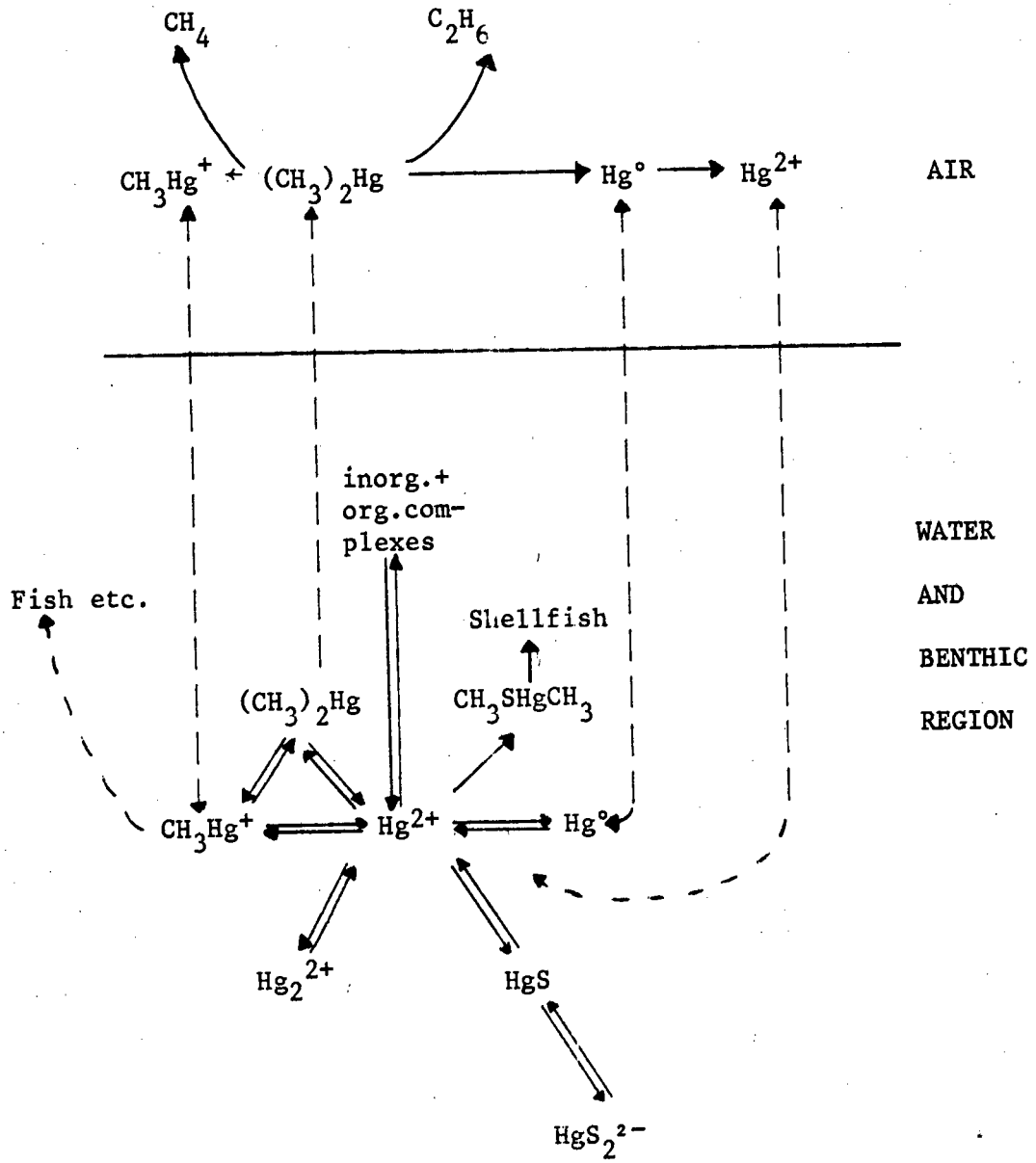


Fig. 5.1 Major transformations of mercury in the environment

a means of detoxification in metal-loaded environments. By such means the bacteria may appear "resistant" to metal compounds. The role of plasmids in conferring such resistance and the linkage with antibiotic resistance is well documented (Mietz & Sjogren 1982; Foster 1983; Weiss et al 1977).

The environmental significance of bacterial bioaccumulation and biotransformation may be briefly summarised:

(a) The resultant detoxification of the environment may be beneficial to other species.

(b) Biotransformed compounds may be more or less toxic than the original.

(c) Bioaccumulation can result in metal transfer to higher trophic levels.

(d) Bioaccumulation by bacteria, particularly those resident on estuarine plants, can effectively immobilise metals.

(e) Proliferation of metal resistant bacteria can alter the microbial ecology of an environment with subsequent disturbances in nutrient cycling and detrimental effects on higher trophic levels.

(f) Bioaccumulating and/or biotransforming micro-organisms, that harbour plasmids, may confer antibiotic resistance, via these plasmids, to pathogenic bacteria.

Metal/microbe interactions are therefore of fundamental significance to both man and his environment.

It was beyond the scope of the present study to undertake detailed investigations into biotransformation, bioaccumulation or plasmid determined resistance mediated by bacteria. However, limited tests were performed to determine if there was any evidence of biotransformation or bioaccumulation of metals by bacteria isolated from Eastern Cape sediments.

5.2 MATERIALS AND METHODS

5.2.1 Media

All media used are listed in the Appendix.

5.2.2 Biotransformation of $CdCl_2$

E. coli (isolated from Papenkuils River sediment) was inoculated into 5 tubes containing 1% tryptone water and $CdCl_2$ at varying concentrations (1,0; 2,5; 5,0; 7,5 and 10,0 µg Cd/ml). Uninoculated tubes of 1% tryptone with $CdCl_2$ (at the same concentrations) were included as control. All tests were carried out in duplicate.

After 18 h at 38°C the contents of the tubes were added to distilled water and the pH adjusted to 8,5. This mixture was then shaken with 25 ml chloroform for 5 min and allowed to stand. The

chloroform layer separated out and was transferred to an Erlenmeyer flask and evaporated to dryness with 1 ml of concentrated redistilled nitric acid. This method of extraction of organically bound metal compounds is a modification of that described by Burton (1978). The same sample that had been chloroform extracted was now extracted using NaDDC-Chloroform (Watling & Watling 1976). The chloroform residues were again evaporated to dryness with 1 ml concentrated redistilled nitric acid.

Metal content of each residue was determined by atomic absorption spectroscopy (AAS) after re-constitution in 2 ml 10% nitric acid.

5.2.3 Initial bioaccumulation experiment

Estimation of bacterial/metal mass: Millipore 45 µm (45 mm) filters were preweighed after drying at 50°C for 3 h and cooling by dessication at 20°C for 1 h. After incubation each metal amended media, either with or without bacteria, was filtered, using a sterile Seitz filter apparatus, through a preweighed filter. These filters were allowed to dry at 20°C

After 3 h drying at 50°C and cooling under dessication at 20°C they were reweighed.

Estimation of metal uptake by bacteria:
cultures of sediment bacteria were stored on tryptone soya agar prior to identification, where

applicable, using the API 20E system. Two strains of each of the following, Bacillus, Pseudomonas, Klebsiella, Escherichia, Aeromonas and Enterobacter were inoculated on MacConkey agar and nutrient agar to determine purity. A few colonies from each plate were inoculated into 1 % tryptone water to obtain vigorously growing cultures. Tryptone glucose broth was prepared in 50 ml aliquots and stock metal solutions added to achieve final concentrations as individual elements of 30 µg Pb/ml ($PbCl_2$); 25 µg Se/ml (SeO_2); 100 µg As/ml (Na_2AsHO_4). Bacterial cultures (0,5 ml) were added to each of the metal amended broths prior to incubation at 30°C for seven days. Controls of uninoculated metal-amended media for each metal under test, and inoculated tryptone-glucose broth without metals, for each bacterial strain were included. After incubation each broth was filtered through preweighed Millipore 45 µm filters prior to drying and reweighing. The filter and residue were digested with 5 ml conc. nitric acid and allowed to evaporate to dryness on a hot plate. Five millilitres of 10% nitric acid were added to the cooled residue and the samples left in sealed vials prior to the determination of metal content.

5.2.4 Second bioaccumulation experiment

Estimation of metal uptake by bacteria: A broth

containing 0,1% tryptone and 0,1% glucose in 25% filtered sea water was prepared and $PbCl_2$ was added to give a final concentration of 30 µg Pb/ml The medium was added in 20 ml aliquots to each of six bottles and sterilised.

An overnight culture of E. coli in 1% tryptone broth (organism No. 8 from the previous experiment) was added to each metal-amended broth (0,2 ml). After incubation at 30°C for 5 days the cultures were washed by centrifuging at 3000 rpm for 10 min. This was repeated twice using 25% sterile sea water as the washing fluid. The deposits were pooled and 5 ml digested with conc. nitric acid. After evaporation and cooling the residue was dissolved in 5 ml 10% nitric acid prior to metal content determination using atomic absorption spectroscopy (AAS). An uninoculated control was included and this was treated as for the inoculated media.

Estimation of bacterial biomass: The washed deposits of the six inoculated metal-amended media were pooled and mixed well. Dilutions of this deposit were made, using 25% sterile sea water to achieve a range of dilution 10^3- 10^8/ml. MacConkey agar plates were surface spread with 0,1 ml of each dilution prior to incubation at 37°C for 18 h.

The tests were done in triplicate. Dilutions showing between 30-300 colonies per plate were used to estimate the total viable count. The total biomass was estimated from the formula (Rodina 1972)

$$\frac{0,25\ \pi\ d^2h\ x\ t}{1000} = M$$

where d = average width of one bacterium (taken arbitarily as 0,5 µ)

h = average length of one bacterium (taken arbitarily as 3,0 µ)

t = No. of thousands of cells/ml^{-1}

M = Biomass in mgm/l.

5.3 RESULTS

5.3.1 Biotransformation of $CdCl_2$

The percentage organic cadmium levels, present after incubation, were calculated from the metal residue concentrations determined by AAS.

The results are shown graphically in Figure 5.2.

The results clearly indicate the marked loss of ionic inorganic cadmium in the inoculated system compared to the uninoculated control.

5.3.2 Bioaccumulation

Initial Experiment: This experiment was designed as a screening procedure to find suitable organisms

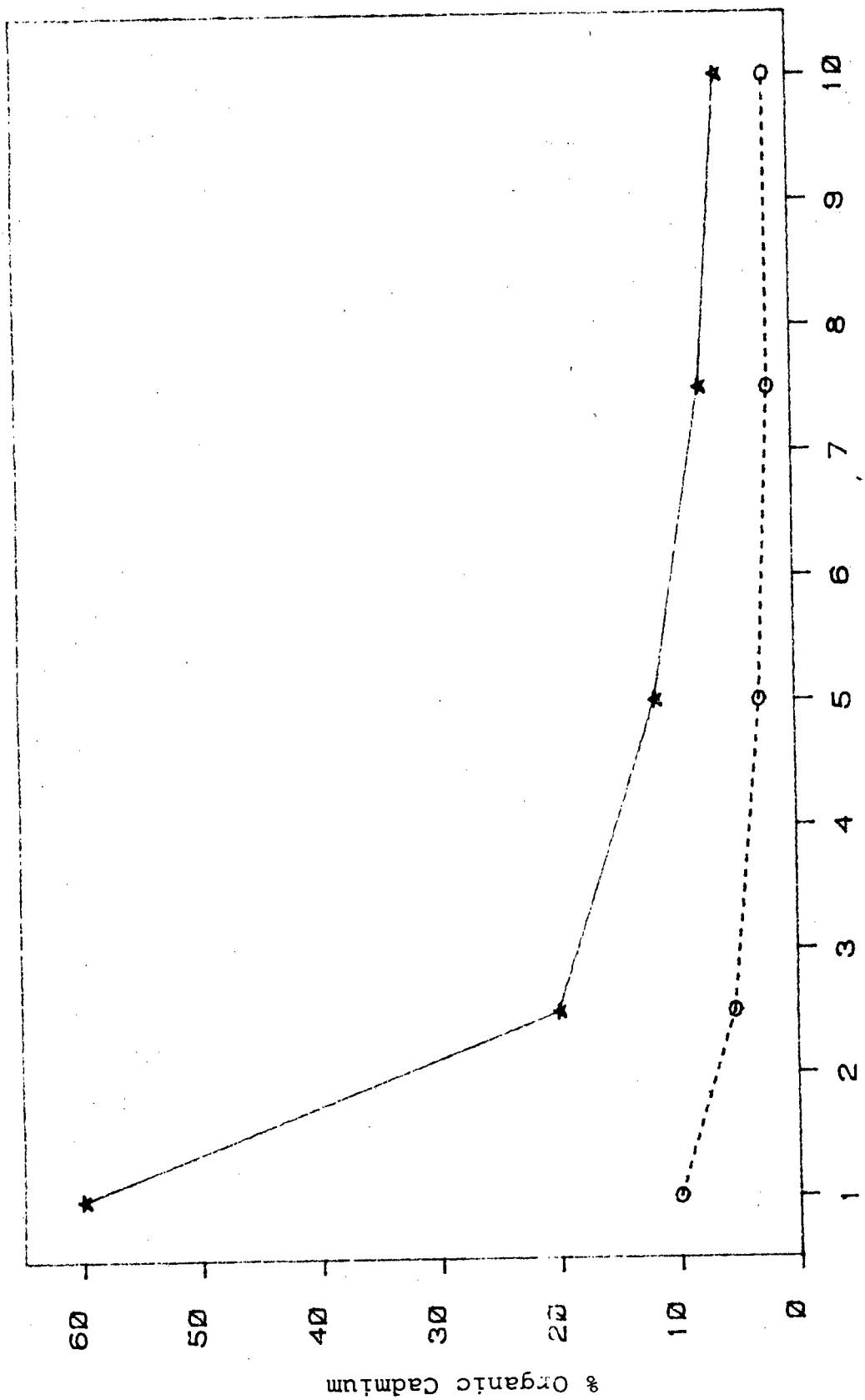


Fig. 5.2 Biotransformation of cadmium chloride by Escherichia coli

for subsequent bioaccumulation experiments. The results of metal uptake (lead, selenium, arsenic) by heterotrophic sediment bacteria are shown in Table 5.1. Fairly high levels of accumulation by most bacterial species was evident for selenium and lead, but not for arsenic. The weights of the bacteria/metal residues varied (Table 5.2). These weights must be assessed according to the amount of metal accumulated and its relative weight. This method of measuring biomass was therefore subsequently discarded.

From these results strain No.8 (E. coli) was chosen for a subsequent, more defined test, to measure uptake of $PbCl_2$.

Second Bioaccumulation Experiment: These results are listed below and they demonstrate the ability of marine heterotrophic bacteria to accumulate large quantities of lead from the surrounding media. The results shown are average of duplicate tests.

Bacterial biomass and Pb content of pellet

Pb content mg ml^{-1}	Total viable count ml^{-1}	Biomass mg ml^{-1}	Pb. conc. mg/ mg bacteria mass
0,0395	88×10^5	0,0052	7,6

TABLE 5.1

Uptake of Pb, Se and As by heterotrophic bacteria

Species	Strain No	Total metal uptake(µg) Pb*	Se*	As*
Klebsiella oxycota	1	52,5	80,0	2,0
	2	710,0	130,0	0,5
Bacillus sp.	3	35,5	6,0	0,6
	4	40,5	20,0	0,3
Pseudomonas sp.	5	37,0	600,0	1,0
	6	83,0	155,0	0,6
Escherichia coli	7	735,0	97,5	1,1
	8	740,0	115,0	0,4
Aeromonas hydrophila	9	565,0	60,0	0,8
	10	545,0	110,0	0,4
Enterobacter cloacae	11	156,0	425,0	1,1
	12	166,0	240,0	3,6

*Total input was 1500 µg Pb, 1250 µg Se and 500 µg As.

TABLE 5.2

Weight (mg) of bacteria + metal residues

Strain No	Media - no metals	As media	Se media	Pb media
1	0,6	2,0	0,1	1,8
2	0,01	0,1	0,8	2,3
3	(a)	0,2	(a)	(a)
4	(a)	0,7	(a)	(a)
5	2,0	2,1	2,8	2,3
6	2,0	1,5	1,6	2,1
7	0,3	1,1	0,9	2,4
8	1,6	0,2	0,7	2,4
9	0,8	2,2	0,9	1,6
10	0,9	0,5	0,6	1,1
11	3,6	1,6	1,4	1,0
12	1,3	2,6	1,9	0,9

(a) = no growth

5.4 DISCUSSION

The results of the biotransformation experiment indicated that in the uninoculated system approximately 95% of the added cadmium remained as ionic inorganic compounds. In the inoculated system up to 60% of the cadmium was removed by the simple chloroform extraction and obviously had, in some way, been biotransformed. The exact nature of the biotransformation mechanism and the end products can, however, only be established using a gas chromatography/mass spectrometric technique which was beyond the scope of the present study.

Metal uptake experiments demonstrated the ability of Gram negative heterotrophic sediment bacteria to accumulate lead and selenium. The amount of metal accumulated varied with bacterial species but results from preliminary tests indicated a 49% uptake of lead by E. coli and a 48% uptake of selenium by Pseudomonas species. The average uptake for lead was 21,5% for all the bacterial strains tested and 25,3% for the Gram negative strains only. In the case of selenium an average of 13,6% uptake by all the bacterial species was found and this was increased to 16,1% when only the Gram negative results were used in the calculation. The apparent lack of accumulation by arsenic may be due to the formation of volatile arsenic compounds (methylarsines) (Klumpp 1980) and subsequent loss into the atmosphere.

Glucose was added to the basal media in order to induce capsule formation by the bacteria. By this means it was

hoped to increase metal uptake (Dunn & Bull 1983).

In the second bioaccumulation experiment lead was incorporated into a dilute sea salts media in order to more closely approximate estuarine conditions. Dry weight, as a measure of biomass, was discarded in favour of total viable count/mass calculations. However, this value was not absolute as an 'average size bacterium' was an arbitary figure only. Despite the limitations of the tests the results clearly indicate the ability of the environmental bacteria to accumulate lead (7,6 mg/mg bacterial mass). The implications for the increased use of bacteria either in metal-ore leaching processes (Förstner 1979) or metal removal from toxic effluents (Remacle & Houba 1983) are apparent. An understanding of metal/microbe interactions with special reference to the effects of biotransformed compounds, is essential in pollution control. The experiments indicated that bacteria capable of biotransformation and bioaccumulation of metals are easily isolated from marine sediments. Therefore detailed further studies of such interactions involving all toxic elements and including aerobic and anaerobic as well as autotrophic and heterotrophic bacteria, are essential to environmental management procedures.

5.5 SUMMARY

The ability of heterotrophic bacteria isolated from E. Cape sediments, to bioaccumulate and biotransform metal compounds was investigated. The results indicated that several

different bacterial species were capable of accumulating metal compounds and that biotransformation was also effected. It was shown that the Gram negative bacteria tested accumulated lead and selenium in greater amounts than Gram positive organisms. Arsenic was not accumulated by any of the strains under test.

CHAPTER 6

6.1 CONCLUSION AND GENERAL DISCUSSION

The aim of this study was to determine metal/bacterial interactions with special reference to the marine sediments of the Eastern Cape. The term metal or metal compound has been used throughout the text in place of "heavy metal" as this term is scientifically imprecise.

Fundamental to the study was the development of a rapid, reliable method to determine bacterial susceptibility to a wide range of potentially toxic metal compounds. Modifications were made to the disc diffusion method for antibiotic susceptibility testing. This modified agar diffusion method gave excellent results for all metals tested with the exception of lead. Diffusion-curve characteristics demonstrated the binding of lead by media constituents. From subsequent trials of several low nutrient media a suitable media for lead toxicity testing was found.

Media-metal content levels were estimated in relation to diffusion from the point source using atomic absorption spectrophotometry. The results of regression analysis of the data so obtained were used to prepare standard graphs from which MIC levels could be determined.

The modified agar diffusion method was used to determine metal-susceptibility patterns of several different strains

of environmental bacteria. The results indicated that, in general, Gram positive bacteria were more sensitive to metal compounds. Zinc was the only metal that exhibited equal toxicity to both Gram negative and Gram positive bacteria.

Axenic cultures of E. coli from metal-polluted and unpolluted sites were tested for metal susceptibility using agar diffusion. The results indicated that background levels of metals in Eastern Cape marine sediments were not related to MIC values determined for the bacteria. Similarily, from antibiotic susceptibility tests of the same isolates, it appeared that there was no co-selection for antibiotic resistance. It is postulated that the metal levels of these sites are not sufficiently high to exert selective pressure for metal resistance.

The toxicity of effluents from different point sources, was determined by agar diffusion. To achieve maximum sensitivity it was necessary to modify the test media. The results indicated that, with this modification, the detection of metal pollution in effluents and the determination of relative toxicity at set time intervals can be both rapid and simple. The method has a unique application to pollution monitoring of industrial effluents.

The results of tests for synergism and antagonism demonstrated that variations occurred according to both the metal and the bacterial species under test. However, the

findings were in agreement with other workers who had used either procaryotic or encaryotic organisms as the test species (Watling & Watling 1982a; Olson & Thornton 1981; Anderson & Weber 1975). There were several results from this study for which equivalent literature citations could not be found. The use of agar diffusion as a screening procedure for synergistic and antagonistic effects of metals would be of considerable value in future investigations of metal/microbe interactions.

The heterotrophic sediment bacteria of the Eastern Cape were shown to have the ability to both accumulate and transform toxic metal compounds. The use of metal-amended media and subsequent filtration techniques was found to be satisfactory for determination of metal uptake by bacteria. The method could be further adapted to demonstrate the effects of bacterial accumulation of metals at higher trophic levels.

The modified agar diffusion method as presented in this study has considerable application in determining metal/bacterial interactions. Its use for both effluent toxicity testing and environmental metal-susceptibility tests has been demonstrated.

Comparison of the results of this study with those in the literature demonstrates the need for the establishment of standardised toxicity testing methods and a rationale for the interpretation of results.

APPENDIX

GENERAL

(a) All sterilisation was done by autoclaving at 121°C for 15 min at 15 lbf/inch² unless otherwise stated.

(b) All techniques for agar diffusion and tube dilution and bacterial maintenance were carried out under aseptic conditions unless otherwise stated.

(c) All glassware was held in 10% HCl prior to washing to eliminate adsorption of metals onto glass surfaces.

(d) All distilled water was deionized and glass distilled.

NUTRIENT AGAR - MEDIA

		(g/l)
Lablemco Powder	(Oxoid L29)	1
Yeast Extract	(Oxoid L21)	2
Peptone	(Oxoid L37)	5
Sodium chloride		5
Agar No. 3	(Oxoid L13)	15
pH 7,4		

NUTRIENT BROTH

		(g/l)
Lablemco Powder	(Oxoid L29)	1
Yeast Extract Powder	(Oxoid L21)	2
Peptone	(Oxoid L37)	5
Sodium chloride		5
pH 7,4		

MACCONKEY AGAR

		(g/l)
Peptone	(Oxoid L37)	20,0
Lactose		10,0
Bile Salts No.3	(Oxoid L55)	5,0
Sodium chloride		5,0
Neutral Red		0,075
Agar No. 3	(Oxoid L13)	12,0
pH 7,4		

TRYPTONE WATER

		(g/l)
Tryptone	(Oxoid L42)	10,0
Sodium chloride		5,0
pH 7,2		

MACCONKEY PURPLE BROTH

		(g/l)
Peptone	(Oxoid L37)	20,0
Lactose		10,0
Bile Salts	(Oxoid L55)	5,0
Bromocresol purple		0,015
pH 7,4		

MACCONKEY PURPLE AGAR

		(g/l)
Peptone	(Oxoid L37)	20,0
Lactose		10,0
Bile Salts	(Oxoid L55)	5,0
Bromocresol purple		0,015
pH 7,4		
Agar No.3	(Oxoid L13)	12,0

TRYPTONE SOYA AGAR

		(g/l)
Tryptone	(Oxoid L42)	15
Soya Peptone	(Oxoid L44)	5
Sodium chloride		5
Agar No. 3	(Oxoid L13)	15
pH 7,3		

SIMMONS CITRATE MEDIA

	(g/l)
Magnesium sulphate	0,2
Ammonium dihydrogen phosphate	0,2
Sodium ammonium phosphate	0,8
Sodium citrate, tribasic	2,0
Sodium chloride	5,0
Bromo-thymol blue	0,08
pH 7,0	

ROBERTSON'S ARTIFICIAL COOKED MEAT MEDIA

		(g/l)
Peptone	(Oxoid L37)	10
Lablemco powder	(Oxoid L29)	10
Textured soya protein		30
Sodium chloride		5
pH 7,0		

1,2% NOBLE AGAR MEDIA

		(g/l)
Noble agar		12
Tryptone	(Oxoid L42)	1
Sodium chloride		5
pH 7,0		

TRYPTONE GLUCOSE MEDIA

		(g/l)
Tryptone	(Oxoid L42)	1
Glucose		1
Filtered sea water	(250 ml)	

25 ml aliquots added to MacConkey bottles.

BRILLIANT GREEN BILE BROTH

		(g/l)
Peptone	(Oxoid L37)	10,0
Lactose		10,0
Ox Bile (purified)		20,0
Brilliant green		0,0133
pH 7,4		

KOVACS INDOLE REAGENT

Paradimethylaminobenzaldehyde	5 g
Amyl alcohol	75 ml
Conc. hydrochloric acid	25 ml

NADDC SOLUTION

Sodium acetate trihydrate (AR)	246 g
Distilled water	500 ml
Glacial acetic acid	11 ml
pH 9-10 with NH_3 solution	
Sodium diethyldithiocarbamate	50 g
Distilled water up to	1000 ml
pH 9,0	

Extract three times with chloroform to remove any residual metal.

SOLUTIONS FOR STANDARDISATION OF ATOMIC ABSORPTION

The nitrates of chromium, nickel, cobalt, zinc cadmium, copper, lead, arsenic, selenium and manganese and the chlorides of mercury were prepared as a top standard at a concentration of 1 000 µg/ml. Methyl mercuric chloride was prepared in absolute alcohol at 1000 µg/ml. Doubling dilutions were made of each standard to provide a calibration series. With the exception of methyl mercuric chloride all standared were prepared in 10% nitric acid.

INCUBATOR

Incubation of cultures through was in a Memmert Series B- incubator (operating - sensitivity $\underline{C}$ 0,5%, Maximum temperature (120°C) Operating - uniformity $\underline{C}$ 1,5% of the maximim temperature) and relative humidity 25%.

PETRI-DISHES

Promex sterile disposable (90 mm) Petri-dishes were used throughout.

REFERENCES

ANDERSON, P.D. and WEBER, L.J. (1975)
The toxicity to aquatic populations of mixtures containing certain heavy metals.
Proceedings of the International Conference on Heavy Metals in the Environment, Toronto 1975, Edinburgh CEP Consultants, pp 933-953.

BABICH, H. and STOTSKY, G. (1978)
Effects of cadmium on the biota: Influence of environmental factors.
Adv. Appl. Micro. 23: 55-117.

BAKIR, F., DAMLUJI, S.F., AMIN-ZAKI, L., MURTADHAM, K.A. ALI-RAWI, N.Y., JIKRITI, S., DHAHIL, H.I., CLARKSON, T.W., SMITH, J.C. AND DOHERTY, R.A. (1973)
Methylmercury poisoning in Iraq.
Science 181: 230-241.

BALDRY, M.G.G. and DEAN, A.C.R. (1980)
Copper accumulation by E. coli strain FE12/5. 2. Uptake by resting organisms
Microbios Letters 15: 105-111.

BARKAY, T., OLSON, B.H. and COLWELL, R.R. (1979)
Heavy metal transformation mediated by estuarine bacteria.
Proceedings of the International Conference on Heavy metals in the Environment, London 1979, Edinburgh, CEP Consultants, pp 356-362.

BARTLETT, L. and ROBE, F.W. (1974)
Effects of copper, zinc and cadmium on Salanastrum capricornutum.
Wat. Res. 8: 179-185.

BAUER, A.W., KIRBY, W.M.M., SHERRIS, J.C. and TURCK, M. (1965)
Antibiotic susceptibility testing by a standardised single disc method.
Am. J. Clin. Path. 45: 493-496.

BAUER, N.J., SEIDLER, R., and KNITTEL, M.D. (1981)
A simple, rapid bioassay for detecting effects of pollutants on bacteria.
Bull. Environ. Contam. Toxicol. 27: 577-582.

BISOGNI, J.Jr. (1973)
Kinetics of microbially medially mediated methylation of mercury in aerobic and anaerobic aquatic environments.
Unpublished PhD thesis, Cornell University. 180p.
Ann Arbor, University Microfilms.

BRAMAN, R. and FOREBACK, C.C. (1973)
Methylated forms of arsenic in the environment.
Science 182: 1247-1249.

BROWN, A.E. and NEWELL, R.C. (1972)
The effect of Cu and Zn on the metabolism of the mussel Mytilus edulis.
Mar. Biol. 16: 108-118.

BURT, S.J. and WOODS, D.R. (1976)
Evolution of transferable antibiotic resistance in coliform bacteria from remote environments.
Antimicrobiol. agents & Chemotherap. 10(3): 567-568.

BURTON, J.D. (1978)
Behaviour of some trace chemical constituents in estuarine waters.
Pure and Appl. Chem. 50: 385-393.

BUSCH, A.W. (1983)
Bioassay technique for relative toxicity in water pollution control.
Water Poll. Contr. Fed. J. 54(7): 1152-1154.

CALABRESE, A., COLLIER, R.S., NELSON, D.A. and McINNIN, J.R. (1973)
The toxicity of heavy metals to embryos of the American oyster Crassostrea virginica.
Mar. Biol. 18: 162-166.

CHAPMAN, G.A. (1978)
Toxicities of Cd, Cu, Zn to juvenile stages of Chinook Salmon and Steelhead.
Trans. Am. Fish. Soc. 107(6): 841-847.

CHAU, T.K., WONG, P.T.S., SILVERBERG, B.A., LUXON, P.L., and BENGERT, G.A. (1976)
Methylation of selenium in the aquatic environment.
Science 192: 1130-1131.

CHERDYNTSEVA, L.M. (1982)
Toxic effects of zinc on Pseudomonas fluorescens.
Hydrobiologia 18(9): 88-90.

COLLWELL, R.R., BERK, S.G., SAYLER, G.S., NELSON, D.J. and ESSER, J.M. (1975)
Mobilization of mercury by aquatic microorganisms.
Proceedings of the International Conference on Heavy Metals in the Environment, Toronto 1975, Edinburgh CEP Consultants pp. 831-843.

CONNER, P.M. (1972)
Acute toxicity of heavy metals to some marine larvae.
Mar. Poll. Bull. 3: 190-193.

DAMYANOVA, A. (1984)
Study of the toxic effects of Cd and Cu on the metabolic processes of bacterial communities.
J. Radioanal. Chem. 77(1): 241-245.

DEVANAS, M.A., LITCHFIELD, C.D., MCCLEAN, C. and GIANNI, J. (1980)
Coincidence of cadmium and antibiotic resistance in New York Bight Apex benthic microorganisms.
Mar. Poll. Bull. 11(9): 264-269.

DUNN, G.M. and BULL, A.T. (1983)
Bioaccumulation of copper by a defined community of activated sludge bacteria.
Europ. J. Appl. Microbiol & Biotech. 17: 30-34

DUXBURY, T. (1981)
Toxicity of heavy metals to soil bacteria.
FEMS Microbiol Letters 11: 217-220.

ERICSSON, H.M. and SHERRIS, J.C. (1971)
Antibiotic sensitivity testing. Report of an international collaborative study.
Acta Pathologica et Microbiologica Scandinavica Section B, Supp. 217: 1-90.

FLEISCHER, M. (1973)
Natural sources of some trace elements in the environment. Cycling and control of metals.
U.S. Environ. Protection Agency. Proc. Environ. Resources Conference 1972, pp. 3-10.

FÖRSTNER, U. (1979)
Mobilization of heavy metals by microbial activity.
Metal Pollution in the Aquatic Environment, Springer-Verlag, pp 265-270.

FOSTER, T.J. (1983)
Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria.
Microbiol. Rev. 47(3): 361-409.

FUJIKI, M. (1963)
Studies on the course that the causative agent of Minamata Disease was formed, especially on the accumulation of the mercury compound in the fish and shellfish of Minamata Bay.
J. Kunmanoto. Med. Soc. 39: 494.

FURATAMI, A. and RUDD, J.W. (1980)
Measurement of Hg methylation in Lake water and sediment samples.
Appl. Environ. Microbial. 40(4): 770-776.

FURMANSKA, M. (1979)
Effect of copper, zinc and iron on the biotic components of aquatic ecosystems.
Pol. arch. hydrobiol. 26 (1-2): 213-220.

GADD, G.M. and GRIFFITHS, A.J. (1978)
Micro-organisms and heavy metal toxicity.
Micro. Ecol. 4: 303-317.

GOULDER, R., BLANCHARD, A.S., SANDERSON, P.L. and WRIGHT, B. (1979)
A note on the recognition of pollution stress in populations of estuarine bacteria.
J. Appl. Bact. 46: 285-289.

GOYAL, S.M., GERBA, C.P. and MELNICK, J.L. (1979)
Transferable drug resistance in bacteria of coastal canal water and sediment.
Wat. Res. 13: 349-356.

GRAY, J.S. (1974)
Synergistic effects of three heavy metals on growth rates of a marine cilliate protozoa.
Pollution & Physiology of Marine Organisms, Eds. Vernberg, F.J. and Vernberg, W.B., Academic Press. pp. 465-488.

GRIFFITHS, A.J., HUGHES, D.E. and THOMAS, D. (1974)
Some aspects of microbial resistance to metal pollution.
Minerals in the Environment. Ed. Jones, M.J. pp. 387-394.

GUAY, R. and SILVER, M. (1977)
Ferrous iron oxidation and uranium extraction by Thiobacillus ferrooxidans.
Biotech. Bioeng. 19: 727-740.

GUTHRIE, R.K. and SINGLETON, F.L. (1977)
Aquatic bacterial populations and heavy metals. Part 2 - Influence of chemical content of aquatic environments on bacterial uptake of chemical elements.
Wat. Res. 11(8): 643-646.

HALLAS, L.E. and COONEY, J.J. (1981)
Tin and tin-resistant microorganisms in Chesapeake Bay.
Appl. & Environ. Micro 41(2): 446-471

HALLAS, L.E., THAYER, J.S. and COONEY, J. (1982)
Factors affecting the toxic effect of tin on estuarine microorganisms.
Appl. & Environ. Microbiol. 44(1): 193-197.

HARRISON, M.J., WRIGHT, R.T. and MORITA, R.Y. (1971)
Method for measuring mineralisation in lake sediments.
J. Appl. Micro 21: 698-702.

HOUBA, C. and REMACLE, J. (1980)
Composition of the saprophytic bacterial communities in freshwater systems contaminated by heavy metals.
Micro. Ecol. 6: 55-69.

JARVIE, A.W.P., WHITMORE, A.P., MARKALL, R.N. and POTTER, H.R. (1983)
Lead biomethylation, an elusive goal.
Environmental Pollution, (Series B) 6: 81-94.

JENSEN, A., RYSTAD, B. and SIGURD, M. (1974)
Heavy metal tolerance of marine phytoplankton.
I. The tolerance of three algal species to zinc in coastal sea water.
J. Exp. Mar. Biol. 15: 145-157.

JENSEN, A., RYSTAD, B. and SIGURD, M. (1976)
Heavy metal tolerance of marine phytoplankton.
II. Copper tolerance of three species in dialysis and batch cultures.
J. Exp. Mar. Biol. 25: 37-50.

JERNELOV, A. (1975)
Microbial alkylation of metals.
Proceedings of the International Conference on Heavy Metals in the Environment, Toronto 1975, Edinburgh CEP Consultants, pp 845-859.

KEMPSTER, P.L., HATTINGH, W.A.J., and VAN VLIET, H.R. (1980)
Summarized water quality criteria.
Dept. Water Affairs, Forestry & Environ. Conservation Technical Report No. TR108 (1980). pp 1-45.

KLUMPP, D.L. (1980)
Characteristics of arsenic accumulation by the sea weeds Fucus spiralis and Ascophyllum nodosum.
Mar. Biol. 58: 257-264.

KOBAYASHI, S. and LEE, G.F. (1971)
Relation between "Itai-Itai" disease and pollution of river water by cadmium from a mine.
Adv. Water. Poll. Res. Proc. 5th Int. Conf. San Francisco. Hawaii, I-25: 1-7.

KURATA, A., YOSHIDA, Y., KADOTA, H. and TAGUCHI, F. (1977)
Distribution of Ni-tolerant bacteria in water and sediments of the Sea of Aso.
Bull. Jap. Soc. Sci. Fish 43(10): 1203-1208.

LAITINEN, H.A. (1973)
Overview of effects of trace metals.
U.S. Environ. Protection Agency, Proc. Environ. Resources Conf. (1972), pp 41-44.

LIGHTHART, B., BAHAM, J. and VOLK, V.V. (1983)
Microbial respiration in metal-amended soils.
J. Environ. Qual. 12(4): 543-548.

L'VOV, B.V. (1978)
Electrothermal atomization - the way toward absolute methods of atomic absorption analysis.
Spectrochim Acta 33B: 153-193

MARCHETTI, R. (1978)
Acute toxicity of alkyl leads to some marine organisms.
Mar. Poll. Bull. 19: 206-207

MARQUES, A.M., CONGREGADO, F. and SIMON-PUJOL, D.M. (1979)
Antibiotic and heavy metal resistance of Pseudomonas aeruginosa isolated from soils.
J. Appl. Bact. 47: 347-350

MASSMANN, H. (1968)
Vergleich von atomabsorption und atomfluoreszenz in der graphitkuevette.
Spectrochimica Acta Part B, pp 215-226.

MAYFIELD, C.I., INNISS, W.E. and SAIN, P. (1980)
Continuous culture of mixed sediment bacteria in the presence of mercury.
Water, Air & Soil Pollut. 13: 335-349.

MCELROY, L.J. (1983)
Detection of industrial pollutants and toxic chemical wastes in sewage treatment plant influents by use of a biological monitor.
Appl. & Environ. Micro 45(2): 730-732.

MIETZ, J.A. & SJOGREN, R.E. (1982)
Incidence of plasmid-linked antibiotic heavy metal resistant enterics in water-sediment from agricultural and harbour sites.
Water, Air & Soil Pollut. 20: 147-159.

MILLS, A.L. and COLWELL, R. (1977)
Microbiological effects of metal ions in Chesapeake Bay water and sediments.
Bull. Environ. Contam. Toxicol. 18(1): 99-103.

MITRA, R.S., GRAY, R.H., CHIN, B., and MINEAR, R.A. (1975)
Molecular mechanisms of accommodation in Escherichia coli to toxic levels of Cd^{2+}.
J. Bact. 121: 1180-1188

NAKAHARA, H., ISHIKAW, T., SARAI, Y., KONDU, I. and MITSUHASH, S. (1977)
Frequency of heavy metal resistance in bacteria from in-patients in Japan.
Nature 266: 165-167

NELSON, J.D. and COLWELL, R.R. (1975)
The ecology of mercury-resistant bacteria in Chesapeake Bay.
Micro. ecol. 1: 191-218.

NORVAL, E. (1978)
Trace elements in the human context.
Symposium on the Analysis of Biological Materials, Pretoria 1977, Oxford Pergamon Press, pp. 23-32.

OLSON, B.H. and THORNTON, I. (1981)
The development of a bacterial indicator system to assess bioacailability of metals in contaminated land.
Proceedings of the International Conference on Heavy Metals in the Environment, Amsterdam 1981, Edinburgh CEP Consultants, pp 254-258.

OLSON, G.J., IVERSON, W., BRINCKMAN, P. and FREDERICK, E. (1981)
Volatilization of mercury by Thiobacillus ferrooxidans.
Current Microbiol. 5: 115-118.

OVERNELL, J. (1976)
Inhibition of marine algal synthesis by heavy metals.
Mar. Biol. 38: 335-342.

PAN-HOU, H.S.K. and IMURA, N. (1981)
Biotransformation of mercurials by intenstinal micro-organisms isolated from yellow fin tuna.
Bull. Environ. Contam. Toxicol. 26: 359-363.

PARKIN, G.F., SPEECE, R.E., YANG, C.H.J. and KOCHER, W.M. (1983)
Response of methane fermentation systems to industrial toxicants.
J. Wat. Poll. Cont. Fed. 55(1): 44-55.

PATRICK, F.M. and LOUTIT, M. (1976)
Passage of metals in effluents through bacteria to higher organisms.
Wat. Res. 10(4): 333-335.

PHILLIPS, D.J.H. (1977)
The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments - a review.
Environ. Pollut. 13: 281-316.

REISINGER, K., STOEPPLER, M. and NÜRNBERG, H.W. (1981)
Evidence for the absence of biological methylation of lead in the environment.
Nature 291(5812): 228-230.

REMACLE, J. and HOUBA, C. (1983)
Uptake of heavy metals from industrial effluents by microorganisms developed in a biological fluidized bed.
Proceedings of the International Conference on Heavy Metals in the Environment, Heidelberg 1983, Edinburgh CEP Consultants 2: 936-939.

RODINA, A.A. (1972)
Methods in aquatic microbiology.
Eds. Colwell, R.R. and Zambruski, M.S., University Park Press, p. 161.

SEYFRIED, P.L. (1980)
Heavy metal resistance in aquatic bacterial isolates.
Aquatic. Toxicol. ASTM STP. 707: 224-232.

SHARIAT, M., ANDERSON, A.G. and MASON, J.W. (1979)
Screening of common bacteria capable of demethylation of methylmercuric chloride.
Bull. Environ. Contam. Toxicol. 21: 255-261.

SIBLEY , T.H. and MORGAN, J.J. (1975)
Equilibrium speciation of trace metals in fresh water: sea water mixing procedures.
Proceedings of the International Conference on Heavy Metals in the Environment, Toronto 1975, Edinburgh CEP Consultants pp 317-338.

SILVER, S., BUDD, K., LEELY, K.M., SHAW, V.W., HANNOD, D. NOVICK, R.P., WILLSKY, G.R., MALAMY, M.H. and ROSENBERG, H. (1981)
Inducible plasmid-determined resistance to arsenate, arsenite, antimony III in E. coli and S. aureus.
J. Bact. 146(3): 983-996.

SIMON-PUJOL, M.D., ESPUNY TOMAS, M.J., CONGREGADO, F. and MARQUES, A.M. (1980)
Heavy metal tolerance of antibiotic Gram negative bacteria isolated from sea water.
Microbios. Letters 15: 151-157.

SJOGREN, R.E. and PORT, J. (1980)
Heavy metal antibiotic resistanc bacteria in a lake recreational area.
Water, Air and Soil pollut. 15: 29-44.

SLOWICK, J. (1981)
Bioaccumulation of copper and lead by Sphaerotilus natans.
Acta Microbiologica polonica 30(2): 182-193.

SMITH, G.W., KOZUCHI, A.M. and HAYASAKA, S.S. (1982)
Heavy metal sensitivity of sea grass Rhizoplane and sediment bacteria.
Botanica Marina Vol. XXV, pp 19-24.

STERRITT, R.M. and LESTER, J.N. (1980)
Interactions of heavy metals and bacteria.
Sci. Tot. Environ. 14: 5-17.

SUMMERS, A. and SILVER, S. (1978)
Microbial transformations of metals.
Ann. rev. Microbiol. 32: 637-672.

TAN, T.L. (1980)
Effect of long term exposure on sea water and sediment bacteria from heterogenous continuous flow cultures.
Micro. Ecol. 5: 295-311.

TETAZ, T.J. and LUKE, R.K.J. (1983)
Plasmid-controlled resistance to copper in Escherichia coli.
J. Bact. 154(3): 1263-1268.

THOMPSON, G.A. and WATLING, R.J. (1984)
Comparative study of the toxicity of metal compounds to heterotrophic bacteria.
Bull. Environ. Contam. Toxicol. 33(1): 114-118.

THOMPSON, G.A. and WATLING, R.J. (1985)
Rapid assessment of effluent toxicities using E. coli as a bioindicator.
Bull. Environ. Contam. Toxicol. (in press)

TURCK, M., LINDEMEYER, R.I., and PETERSDORF, G. (1963)
Comparison of single disc and tube dilution techniques in determining antibiotic sensitivity of Gram negative pathogens.
Annals. Internal Medicine 58: 56-65.

WATLING, H.R. and WATLING, R.J. (1976)
Preconcentration of extraction techniques for the determination of trace elements in water. 1. Sodium diethyldithiocarbamate-chloroform.
Pretoria, CSIR Special Report FIS 83, 70p.

WATLING, H.R. and WATLING, R.J. (1982a)
Comparative effects of metals on the filtering rate of the brown mussel Perna perna.
Bull. Environ. Contam. Toxicol. 29: 651-657.

WATLING, R.J. (1975)
The determination of Hg at picogram/litre level in water with a micro-wave induced argon plasma emission system.
Anal. Chim. Acta 75: 281-288.

WATLING, R.J. and EMMERSON, W.D. (1981)
A preliminary pollution survey of the Papenkuils River, Port Elizabeth.
Water SA 7(4): 211-215.

WATLING, R.J. and WATLING, H.R. (1982b)
Metal surveys in South African estuaries. I. Swartkops River.
Water SA 8(1): 26-35.

WEISS, A.A., MURPHY, S.D. and SILVER, S. (1977)
Mercury and organomercurial resistances determined by plasmids in Staphylococcus auerus.
J. Bact. 132(1): 197-208.

WILLIS, J.B. (1978)
Development of atomic absorbance spectroscopic techniques in biological analysis.
Symposium on the Analysis of Biological Materials, Pretoria 1977, Oxford Pergamon Press, pp 9-22..

WITTMAN, G.T.W. (1981a)
Catastrophic episodes of metal poisoning.
Metal Pollution in the Aquatic Environment,
2nd Edition, Springer-Verlag, pp 18-25.

WITTMAN, G.T.W. (1981b)
The sources of metal pollution.
Metal Pollution in the Aquatic Environment,
2nd Edition, Springer-Verlag, pp 30-57.

WRENCH, J.J. and ADDISON, R.F. (1981)
Reduction, methylation and incorporation of arsenic into lipids by the marine phytoplankton Dunaliella tertiolecta.
Can. J. Fish. & Aqua. Sci. 38(5): 518-523.

YAMADA, M. and TONOMURA, K. (1972)
Formation of methylmercury compounds from inorganic mercury by Clostridium cochlearium.
J. Ferment. Technol. 50(3): 159-166.

YOUNG, D.R., YOUNG, C.S. and HLAURA, G.E. (1973)
Sources of trace metals from highly urbanised Southern California to the adjacent marine ecosystem.
U.S. Environ. Protection Agency. Proc. Environ. Resources Conf. 1972, pp 21-39.

ZEELIE, C. and MCCARTHY, T.J. (1983)
Antioxidants - Multifunctional preservatives for cosmetic and toiletry formulations.
Cosmetics & Toiletries 98(12): 51-55.