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Comparative Study of the Toxicity of Metal Compounds to Heterotrophic Bacteria

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The study of the interactions of heavy metals and bacteria is fundamental to the understanding of the environmental impact of metal compounds. Bioaccumulation and biotransformation of metals by bacteria, together with co-selection of antibiotic resistant strains in metal-polluted environments, have been reported (Patrick & Loutit 1976; Simon-Pujol et al 1980; Summers & Silva 1978). Agar dilution and tube dilution are two methods commonly used to determine bacterial resistance. 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 microorganism is obviously a major factor in determining the minimum inhibitory concentration (MIC) as is the standardization of the test procedure.

A standardised agar diffusion method for the determination of bacterial susceptibility to Cu and Cd has been described (Thompson & Watling 1983). The method has been applied to the determination of bacterial sensitivity and MIC levels for Zn, As, Mn, Ni, Cr, Hg, Se, Pb and Co using E. coli as the test organism. The results of this comparative study are presented here.

MATERIALS AND METHODS

The agar diffusion method and the preparation of metal-diffusion gradients are described by Thompson & Watling (1984).

Aliquots of inoculated and uninoculated nutrient agar were poured into petri dishes. Metal-impregnated paper discs were placed on the agar surface. After an 18 h (37°C) incubation period, the width of the zones of inhibition of bacterial growth were measured. Strips of agar 2 mm wide were removed sequentially from the plate and digested in nitric acid. The metal concentrations in the sample solutions were determined using atomic absorption spectrophotometry.

The amounts of metals added to the discs were 100 µg of Zn, Mn, Ni, Pb or Co as the metal chlorides; 100 µg Se(dioxide) and Cr (trioxide); 200 µg As(sodium arsenate); 250 µg As(monosodium

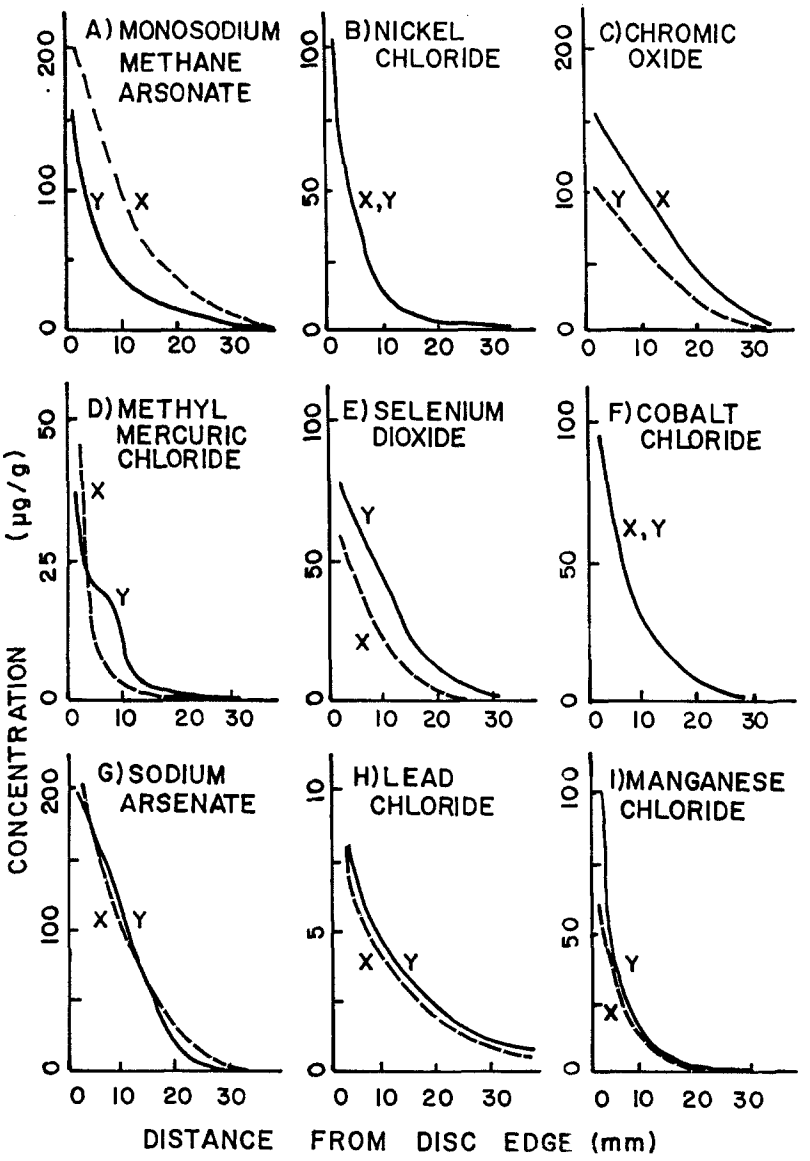

Fig. 1 Diffusion gradients for study compounds (x-uninoculated medium; y - inoculated medium).

methanearsonate (MSMA) and 50 µg Hg(methylmercuric and mercuric chlorides). All tests were carried out in duplicate.

RESULTS AND DISCUSSION

Diffusion gradients for each metal are shown in Figure 1. 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

Table 1: MIC values for the study compounds

Metal Compound	Zone (mm)	Description of Zone	MIC (µg/g)
Zinc chloride	4.0	Clear, slight concentration at outer edge	37
Sodium arsenate	10.1	Clear to 4.0 mm then very slightly diffuse to 10.1 mm	118
MSMA	13.1	Clear to 9.0 mm then very slightly diffuse to 13.1 mm	25
Manganese chloride[1]	No zone		
Manganese chloride[2]			115
Nickel chloride	2.7	Clear, slight concentration at outer edge	88
Chromium trioxide	6.7	Clear, moderate concentration at outer edge and several discrete colonies within inhibition zone	127
Methylmercuric-chloride	25.0	Clear	5.5
Mercuric chloride	13.3	Clear, slight concentration at outer edge	3.5
Selenium dioxide	9.2	Diffuse with less growth up to 5.1 mm	30
Lead chloride[1]	No zone		
Lead chloride[2]	4.3	Clear	38
Cobalt chloride	5.9	Clear	50

(1) Nutrient agar (2) Noble agar

curve and expressed as µg/g metal in the agar. These results are summarized in Table 1.

The diffusion gradients (Fig. 1) indicate that metal compounds 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 nonexponential region of the diffusion curve.

In the cases of Pb and Mn no zones of inhibition of bacterial growth were visible. The Pb diffusion gradient (Fig. 1H) indicated that this metal had diffused poorly through the agar. The Mn diffusion characteristics were also poor (Fig. 1I) 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 diffusion characteristics.

The experiments were repeated for Pb and Mn using various other media. It was found that a 1.2% noble agar (without nutrients) and a heavier inoculum in tryptone broth yielded a good diffusion gradient for lead with a visible zone of inhibition of bacterial growth (Fig. 2A). The diffusion gradient characteristics for manganese improved slightly (Fig. 2B) but no zone of inhibition was visible, confirming that this element is non-toxic.

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. 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.

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 1983). 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 compound.

The MIC is an expression of bacteriostatic action. In order to

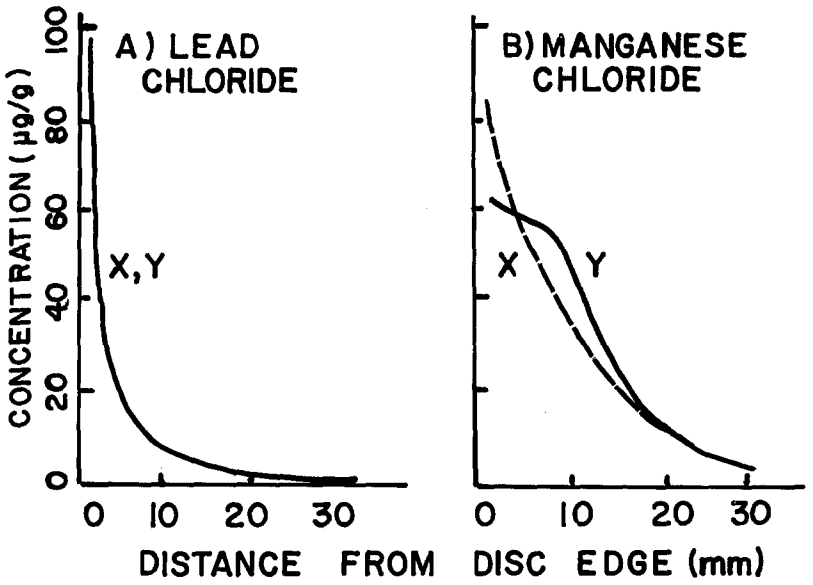

Fig. 2 Diffusion gradients for lead chloride and manganese chloride using noble agar medium (x - uninoculated medium; y - inoculated medium)

assess the bacteriocidal effect of the study compound it is necessary to transfer any 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.

When agar diffusion MIC values are compared with those obtained using tube dilution, it is apparent that the latter technique yields higher results. These differences could be attributed to either an accelerated growth rate in liquid media or the presence of a few resistant cells. There is also the possibility that the metal compound under investigation could form a suspension or precipitate and cloud the solution. This would not only give the appearance of bacterial growth but would also make less metal available to the bacteria.

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.

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.

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, selenium, lead and cobalt. Diffusion gradient graphs prepared for each metal compound define the concentrations of metal available to the 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>MSMA>selenium dioxide>zinc chloride>lead chloride>cobalt chloride>nickel chloride> sodium arsenate>chromium trioxide>manganese chloride.

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