Distinction between Heavy Metal and Organic Toxicity using EDTA Chelation and Microbial Assays

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ABSTRACT

Use of the EDTA chelation test in conjunction with microbioassays for general toxicity (Microtox) and heavy metal toxicity (ß-galactosidase activity) allowed us to identify the presence of heavy metal toxicity and distinguish between cases in which toxicity was due solely to either heavy metals or organic compounds or to a mixture of these chemicals. The technique is effective over a wide range of water hardness and pH. Screening of industrial wastewater samples by the EDTA-microbioassay technique gave results that were consistent with chemical analysis of samples by atomic absorption.

Key words: Microtox, β-galactosidase, heavy metals, toxicity, microbial assays, organics, EDTA

Running head: Toxicity screening by EDTA-microbioassay technique

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INTRODUCTION

The analytical determination of metals, organics, and inorganic nonmetals proceeds by different techniques. Treatment technologies for these classes of compounds also differ. It is therefore advantageous to identify heavy metal toxicity in wastewater samples that are being screened for toxic pollution.

One of the techniques proposed for screening of heavy metal toxicity utilizes XAD resin for removal of organics, followed by passage of the XAD column effluent through cation and anion exchange resins (Walsh and Garnas, 1983). A disadvantage of this technique is that the XAD resin fails to retain polar organics. These polar compounds are then collected along with metals in the subsequent ion-exchange separations.

Another technique, chelation with EDTA, has been used to neutralize heavy metal toxicity in algal bioassays (Couture *et al.*, 1985) and, more recently, the EPA phase I fractionation procedures (Mount and Anderson-Carnahan, 1987). Most heavy metals exist in solution as multivalent cations and are thus susceptible to chelation by EDTA. Disadvantages of this technique are that EDTA does not neutralize the toxicity of metals that exist as anions in solution (e.g., AsO_4^{3-}, $Cr_2O_7^{2-}$), and might chelate cationic organics.

Biological or biochemical assays that are sensitive to particular groups of chemicals should be developed. Heavy metals have broad non-specific inhibitory effects on enzymes, whereas organic compounds tend to be specific in their modes of inhibition. For example, Dutton *et al.* (1988) have shown that ß-

galactosidase activity was generally sensitive to heavy metals but was insensitive to all organic compounds tested. Another microbial assay, Microtox, is sensitive to most heavy metals as well as to a broad array of toxic organic compounds, however.

The purpose of the present research was to use ß-galactosidase activity and Microtox in parallel as toxicity endpoints for the EDTA chelation technique in order to distinguish between heavy metal and organic toxicity. This work was carried out using pure compounds and industrial wastewater samples from the City of Jacksonville, Florida.

MATERIALS AND METHODS

Model Toxicants

The model toxicants included five heavy metals [cadmium ($CdSO_4$), copper ($CuSO_4$ $5H_2O$), zinc ($ZnSO_4$), lead ($PbNO_3$), mercury ($HgSO_4$)] and eight organics [pentachlorophenol (PCP), hexachlorocyclohexane (lindane), phenol, sodium dodecyl sulfate (SDS), benzene, formaldehyde, tetrachloroethylene, and n-(phosphonomethyl)glycine (glyphosate)]. Potassium cyanide was used to represent an inorganic nonmetal, sodium arsenate and potassium dichromate were used as anionic metals, and diquat dibromide was used as a cationic organic. All chemicals were ACS grade or higher.

Stock solutions of PCP and lindane were prepared with methanol. Stock solutions of the other chemicals were prepared with MilliQ water (Millipore Corp., New Bedford, MA). Working solutions of the metals employed in the determination of EC_{50}s

were prepared using moderately hard, reconstituted water [$CaSO_4$, 51.1 mg/L; $NaHCO_3$, 96.0 mg/L; $MgSO_4$, 60.0 mg/L; KCl 4.0 mg/L (Peltier and Weber, 1985)]. Working solutions of metals and organics used in the EDTA chelation tests were prepared in filtered (Whatman GF/C, 1.2 μm effective pore size) nontoxic secondary effluent from the University of Florida's wastewater treatment plant. Final methanol concentrations in the PCP and lindane working solutions were 1% or less, within the nontoxic concentration range for Microtox and ß-galactosidase activity.

Wastewater Samples

A total of 10 grab samples were obtained from the Buckman collection system, Jacksonville, FL. The samples were stored at 4°C for up to 3 days prior to analysis.

EDTA Chelation Procedure

Volumes of 0.25, 0.5, 0.75, and 0.95 mL of EDTA stock solution of a particular concentration (e.g., 0.1 mM) were added to 5 mL of the sample, followed by mixing. The maximum dilution caused by EDTA addition was 16%. This process was repeated using EDTA stock solutions spanning a four-log range of concentrations. For example, if EDTA stocks of 0.1 mM to 100 mM were used, the corresponding range of final EDTA dosages to the samples would be 4.8 μM to 16,000 μM.

Effect of Hardness on EDTA Chelation

The effect of hardness on EDTA chelation was evaluated by carrying out the EDTA chelation test in MilliQ water, moderately

hard, hard, and very hard waters having measured total hardness values of 0, 98, 195, and 254 mg/L as $CaCO_3$, respectively. The EDTA chelation test was also performed on these same waters after they were dosed with 5 mg/L copper. Hardness of the reconstituted water and of the industrial wastewater samples was measured according to the EDTA titration method described in Standard Methods, method 314 B (APHA et al., 1985). ManVer (Hach, Loveland, CO) indicator reagents were used in powder pillow for the titration step.

ß-Galactosidase Activity

Isopropyl-ß-D-thiogalactopyranoside (IPTG) and o-nitrophenylgalactopyranoside (ONPG) were obtained from Sigma. They were prepared at respective concentrations of 0.2% and 0.4% in MilliQ water and sterilized by filtration through a 0.2 μm membrane filter (Gelman, Acrodisc). Sodium phosphate buffer (pH 7.0) contained 1.6% w/v Na_2HPO_4 $7H_2O$ and 0.55% w/v NaH_2PO_4 H_2O. Other reagents employed were 0.1% w/v sodium dodecyl sulfate and 1M Na_2CO_3.

Assays were conducted using the tolC mutant strain of Escherichia coli (EW1b, CGSC #5634, Yale Univ.) (Whitney, 1971). This strain was maintained in 40% glycerol at -15°C. It was grown by inoculating 25 mL LB media [Bacto tryptone, 10 g/L; Bacto yeast extract, 5 g/L; NaCl, 10 g/L (Miller, 1972)] in a 125 mL Erlenmeyer flask with 50 μL of glycerol culture. The bacteria were incubated at 35°C overnight, then diluted with fresh LB media to an absorbance (A_{550}, 1 cm) of 0.3 to 0.4. IPTG was added

to the bacterial cells at a ratio of 1:9 to induce the production of ß-galactosidase. Cell growth was continued to an A_{550} of 0.6 to 0.7. The cells were then exposed to polymyxin B sulfate (Sigma), a polycationic antibiotic, to increase outer membrane permeability. After 10 minutes, the cells were centrifuged to remove unreacted polymyxin and resuspended in 10 mL 0.85% saline (pH 7.0).

A 0.1 mL volume of prepared cells was exposed to 0.9 mL sample for 30 minutes, then 0.8 mL buffer, 0.1 mL SDS and 0.2 mL ONPG were added. Hydrolysis of ONPG by ß-galactosidase releases the yellow colored o-nitrophenol. The reaction was stopped after 10 minutes by adding 1 mL Na_2CO_3. The intensity of the yellow color was determined with a spectrophotometer (Spec 21, Milton Roy Co, Rochester, NY) at 420 nm. Reconstituted, moderately hard water (Peltier and Weber, 1985) or nontoxic secondary effluent was used for controls, as appropriate. All incubations were at 35°C. The degree of inhibition was determined on the basis of measured absorbance values, considering the control to represent 0% inhibition. Blanks with MilliQ water substituted for ONPG were run with each sample. Six replicate tubes were used for the controls and three replicate tubes were used for each toxicant or EDTA concentration.

Microtox

Lyophilized Photobacterium phosphoreum cells (Microbics, Carlsbad, CA) were reconstituted for use in the tests. All assays were carried out at 15°C with a 15-minute contact period.

In definitive assays to determine EC_{50}s, four or more duplicate dilutions were run on each chemical. In the EDTA chelation procedure, Microtox assays were run in duplicate at each EDTA concentration. Data were tabulated and reduced according to the Microtox Operating Manual (Microbics, 1982).

Heavy Metal Analysis

A Perkin Elmer 5000 spectrophotometer was used for analysis in the air-acetylene mode. Samples and standards were acidified and concentrated by boiling in 125 mL Erlenmeyer flasks prior to analysis. The sample concentrations were read directly from the digital output by using the internal standard curve of the instrument. The internal standard curve of the instrument was set by exposing the spectrophotometer detector with several standard concentrations of the metal under investigation. Perkin Elmer Intensitron™ and Fisher Scientific hollow cathode lamps were used for the analysis.

RESULTS

Relative Sensitivity of ß-Galactosidase Activity to Heavy Metal, Organic and Cyanide Toxicity

In order to be a useful adjunct to EDTA chelation, ß-galactosidase activity should be sensitive to most heavy metals and insensitive to most organics and inorganic nonmetals. The effect of a broad group of selected heavy metals and organics, in addition to cyanide, was therefore tested. Each chemical was tested at a concentration 10x higher than its EC_{50} to Microtox. As shown in Figure 1, ß-galactosidase activity was sensitive to

five of the six heavy metals tested. It was insensitive to lead, the metalloid arsenic (in the form of arsenate) and all eight organics.

Neutralization of Heavy Metal Toxicity by EDTA

The effect of EDTA on copper and cadmium toxicity is shown in Figure 2. ß-galactosidase activity and Microtox responded in similar fashion to secondary effluent dosed with 2.0 mg/L (31 μM) copper and 0.6 to 6300 μM EDTA (Figure 2, top). Toxicity was completely neutralized at EDTA dosages of 80 to 200 μM. Higher dosages retoxified the sample.

Microtox did not respond to secondary effluent dosed with 1.2 mg/L (11 μM) cadmium, whereas ß-galactosidase activity was inhibited by 88% at this metal concentration (Figure 2, bottom). Toxicity to ß-galactosidase activity was eliminated at EDTA dosages of 15 to 160 μM. The responses of both microbial assays indicated retoxification at EDTA dosages in excess of 160 μM.

Effect of EDTA on the Toxicity of Organics and Heavy Metal-Organic Mixtures

The effect of EDTA on a secondary effluent dosed with 10 mg/L (34.7 μM) sodium dodecyl sulfate (SDS) is shown in Figure 3a. The percent inhibition of Microtox remained near 100% at all EDTA dosages. The sample was initially nontoxic to ß-galactosidase. It was toxified at EDTA dosages in excess of 240 μM.

Figure 3b shows the effect of EDTA on the toxicity of secondary effluent dosed with a mixture of 2.5 mg/L (38.75 μM) copper and 10 mg/L (34.7 μM) SDS. Toxicity of the solution to ß-

galactosidase activity was neutralized at 50 to 560 μM EDTA, whereas Microtox inhibition remained near 100% over the range of EDTA dosages. A similar pattern of response to EDTA addition was observed in a mixture of 0.6 mg/L (5.5 μM) cadmium and 5 mg/L (17.35 μM) SDS (Fig. 3c).

Influence of Metal Concentration on Test Results

The effect of EDTA on secondary effluent dosed with 56 mg/L (870 μM) copper and on the same solution diluted to 7 mg/L (110 μM) copper is shown in Figure 4a. Toxicity of the dilute copper solution was completely neutralized at EDTA dosages of 100 to 1000 μM. No detoxification was observed with the concentrated solution. Sufficient EDTA (up to 17,800 μM) was added to theoretically detoxify the concentrated copper solution.

Effect of EDTA on a Positively Charged Organic Molecule

Diquat dibromide is a cationic organic molecule which is used as a herbicide in agriculture. Figure 4b shows that EDTA failed to neutralize the toxicity of a sample containing 1000 mg/L (2900 μM) diquat dibromide. Toxicity increased considerably at EDTA dosages in excess of 2000 μM.

Effect of EDTA on the Toxicity of Negatively Charged Heavy Metal Molecules

The effect of EDTA on the toxicity of heavy metals which occur in negatively charged forms is shown in Figures 4c and 4d. Toxicity of sodium arsenate (1335 μM As) to Microtox was slightly decreased by EDTA addition (Fig. 4c). Figure 4d shows that EDTA

failed to neutralize the toxicity of secondary effluent containing potassium dichromate (3365 μM Cr).

Influence of Hardness on Test Results

The effect of hardness on the toxicity of EDTA to Microtox is shown at the top of Figure 5. Little difference was observed in the response of Microtox to EDTA in moderately hard, hard, and very hard waters. EDTA dosages in excess of 1000 μM toxified these samples. MilliQ water was toxified at EDTA dosages exceeding 200 μM.

Figure 5 (bottom) illustrates the effect of hardness and EDTA on the toxicity of 5 mg/L (77.5 μM) copper to Microtox. Toxicity was completely neutralized in the moderately hard, hard and very hard waters at an EDTA dosage of 100 μM. Complete neutralization of copper toxicity in MilliQ water was not observed. Copper in MilliQ water showed higher toxicity than the other water samples at EDTA dosages greater than 100 μM.

Influence of pH on Test Results

The effect of pH on EDTA toxicity to β-galactosidase activity was investigated by the EDTA treatment of copper and cadmium in moderately hard water. The toxicity of EDTA itself in relation to pH was also investigated. Figure 6a illustrates the effect of pH on the detoxification of moderately hard water dosed with 3.5 mg/L (54.25 μM) copper. Complete detoxification was not observed at a pH of 4. At pH 5 the solution was detoxified at 60 μM EDTA. Neutralization of toxicity at pH between 6 and 8 occurred at EDTA dosages of 630 to 1250 μM.

Figure 6b illustrates the effect of increasing pH on the detoxification of moderately hard water dosed with 1.5 mg/L (13.75 μM) cadmium. Toxicity was not neutralized at pH 4 or 5. Detoxification occurred at an EDTA dosage of 100 μM at pH between 6 and 8. The range of EDTA dosage at which toxicity remained absent became broader at higher pH (e.g., 100 to 160 μM at pH 6 versus 100 to 1000 μM at pH 8).

Figure 6c illustrates the effect of pH on the toxicity of EDTA to ß-galactosidase activity in moderately hard water. EDTA is more toxic at the lower pH range.

Wastewater Screening

Use of a microbioassay sensitive to general toxicity (Microtox) and one sensitive only to heavy metals (β-galactosidase activity) in conjunction with EDTA enables the distinction between different types of toxicity. Table I shows possible test patterns and what they indicate.

We screened ten industrial wastewater samples from the Buckman collection system in order to determine if the expected test patterns would be found and to correlate results that indicated the presence of heavy metals with the results of chemical analysis by AA. The results are summarized in Table II.

Samples 1 and 2 were toxic to both microbial assays and the toxicity was completely neutralized by EDTA addition. Sample 3 was toxic to Microtox but nontoxic to ß-galactosidase. The toxicity was completely neutralized by EDTA addition. These results suggest that only cationic heavy metals were responsible

for the observed toxicity. Chemical analysis revealed samples 1 and 2 to contain 1.8 to 1.9 mg/L of nickel and 2.5 to 2.8 mg/L of zinc, as well as lower concentrations of the other metals assayed. Sample 3 contained 0.36 mg/L nickel, smaller concentrations of copper, iron, lead and zinc, and no cadmium.

EDTA neutralized the toxicity of samples 4, 5 and 6 to β-galactosidase but failed to neutralize the toxicity of these samples to Microtox. Each of these samples contained some heavy metals. Sample 5 had the highest concentrations, including 0.58 mg/L copper and 1.52 mg/L nickel. The inability of EDTA to neutralize the toxicity to Microtox suggests that toxic organics were present.

Samples 7, 8, 9 and 10 were highly toxic to Microtox and moderately toxic or nontoxic to β-galactosidase. Toxicity of these samples was not reduced by EDTA addition. These results suggest that organics or cyanide were primarily responsible for the observed toxicity and that any heavy metals present were not readily available. Samples 7, 8 and 9 had high concentrations of nickel, whereas sample 10 was almost free of the metals tested for.

DISCUSSION

The insensitivity of ß-galactosidase activity to organic compounds is in agreement with results in the literature. For example, Dutton et al. (1988) reported that the EC_{50}s of selected organics to ß-galactosidase activity were more than 1000x larger than the EC_{50}s of these compounds to other bioassays (Microtox, Daphnia, fish). The relative insensitivity of ß-galactosidase

activity to organics, metalloids and heavy metals in anionic forms may be attributed to the specific mode of action of these toxicants as opposed to the nonspecific mode of action of heavy metals in cationic form (Christensen et al., 1982). Cationic heavy metals react with sulfur-donor atoms of ß-galactosidase, deactivating the enzyme. In addition, cationic heavy metals can destabilize the conformation of enzymes through the replacement of essential cations (Rudd, 1989).

The range of molar EDTA concentrations that neutralized cadmium and copper toxicity was similar to the range of molar concentrations of these metals in the samples tested, which is consistent with the general observation of a 1:1 molar chelation ratio between heavy metals and EDTA (Fritz and Schenk, 1975).

The toxic effect of elevated EDTA concentrations on β-galactosidase activity and bioluminescence may be attributed to the chelation of essential ions (e.g., Ca^{2+}, Mg^{2+}) that act as cofactors in enzymatic reactions. This is supported by the results of Figure 5 (top) which show that the concentration at which EDTA becomes toxic in MilliQ water is ten times lower than the threshold toxic concentration of EDTA in reconstituted water (containing Ca^{2+} and Mg^{2+}).

The effect of pH on the toxicity of EDTA as demonstrated in Figure 6 is in agreement with the theoretical principles that govern the passage of substances through cell membranes. EDTA becomes nonpolar by protonation at low pH, and this will make its passage through the membrane easier than when the EDTA molecule is in the polar form. EDTA will have the tendency of losing

protons inside the cell (neutral pH conditions) and will chelate cations essential to the proper metabolic cell functions (Rudd, 1989).

Hardness has little effect on EDTA chelation of heavy metals. This may be attributed to the low stability constants of EDTA complexes with Ca^{2+} and Mg^{2+} (Log K values of 10.7 and 8.7, respectively) when compared to the stability constants for EDTA complexes with heavy metals (Dow Chemical, 1982).

The EDTA-microbioassay method for identifying heavy metal toxicity was positive in 7 of the 10 wastewater samples tested. The 3 samples for which this technique was negative contained nickel, lead, and zinc at concentrations larger than 0.5 mg/L. Negative screening results could result from the biological unavailability of heavy metals, due to their adsorption on particulates or chelation by organics or other ligands.

The EDTA-microbioassay technique evaluated in this research is a step forward in the search for simple and rapid toxicity fractionation approaches. The microbial tests for general toxicity (Microtox) and heavy metal toxicity (β-galactosidase) provide complementary information that is useful in conjunction with EDTA to distinguish between heavy metal and organic toxicity. One problem with the method is that EDTA is toxic at high concentration to the microbial assays used. Therefore, a large number of EDTA dosages must be tried to find the one that is large enough to neutralize the heavy metal toxicity but not so high as to toxify the sample. Nontoxic chelating agents or use

of columns containing chelating or cation exchange resins are alternatives that should be investigated in order to make this method faster.

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TABLE I.
Distinction between heavy metal toxicity and other toxicants using the EDTA-microbioassay technique

Initial		Toxicity neutralized by EDTA?		Toxicants present
Microtox	β-galactosidase activity	Microtox	β-galactosidase activity	
Toxic	Toxic	Yes	Yes	Cationic heavy metals
Toxic	Toxic	No	Yes	Cationic heavy metals and organics or CN^-
Toxic	Nontoxic	No	No	Organics or CN^-
Nontoxic	Toxic	No	Yes	Cd^{2+}

TABLE II.
Comparison of Wastewater Detoxification by EDTA Chelation Treatment to Metal Concentrations

Sample no.	Detoxification by EDTA[a]		Metal concentration, mg/L[b]					
	Microtox	ß-gal. act.	Cd	Cu	Fe	Ni	Pb	Zn
1	+	+	0.03	0.05	0.31	1.93	0.07	2.8
2	+	+	nd	0.02	0.26	1.8	0.03	2.5
3	+	NT	nd	0.02	0.04	0.36	0.01	0.09
4	-	+	nd	0.14	1.6	0.18	nd	nd
5	-	+	0.01	0.58	0.32	1.52	0.06	0.18
6	-	+	0.36	0.06	1.7	0.34	1.0	0.36
7	-	NT	0.03	0.02	0.18	0.75	0.16	0.07
8	-	NT	0.11	0.12	0.52	2.66	0.55	0.18
9	-	NT	0.02	0.24	0.23	1.49	0.26	0.97
10	-	NT	0.01	0.03	0.32	0.0008	0.05	0.17

[a] (+) = Detoxification by EDTA, (-) = no detoxification by EDTA, NT = nontoxic.
[b] nd = not detected

Figures

1. **Relative sensitivity of ß-galactosidase activity to heavy metals, cyanide and organics**
2. **Effect of EDTA on heavy metal toxicity to ß-galactosidase activity and Microtox.** Top, Cu^{2+} (31 μM); bottom, Cd^{2+} (11 μM)
3. **Effect of EDTA on the toxicity of SDS and SDS-heavy metal mixtures to β-galactosidase activity and Microtox.** A - SDS (35 μM); B - Cu^{2+} (16 μM) and SDS (17 μM); C - Cd^{2+} (6 μM) and SDS (17 μM)
4. **Effect of EDTA on toxicity of concentrated metal solution, a cationic organic compound, and anionic heavy metal compounds to Microtox.** A - Cu^{2+} at low (108 μM) and high (868 μM) concentrations; B - diquat dibromide (2900 μM); C - sodium arsenate (1340 μM as As); D - potassium dichromate (3370 μM as Cr)
5. **Effect of hardness on the EDTA chelation technique.** Top - EDTA alone; bottom - EDTA added to Cu^{2+} (78 μM) solutions. Toxicity was determined by Microtox. MQ = MilliQ water; MH = moderately hard water; H = hard water; VH = very hard water
6. **Effect of pH on the EDTA chelation technique.** A - Cu^{2+} (54 μM); B - Cd^{2+} (14 μM); C - EDTA alone. Toxicity was determined by β-galactosidase activity.

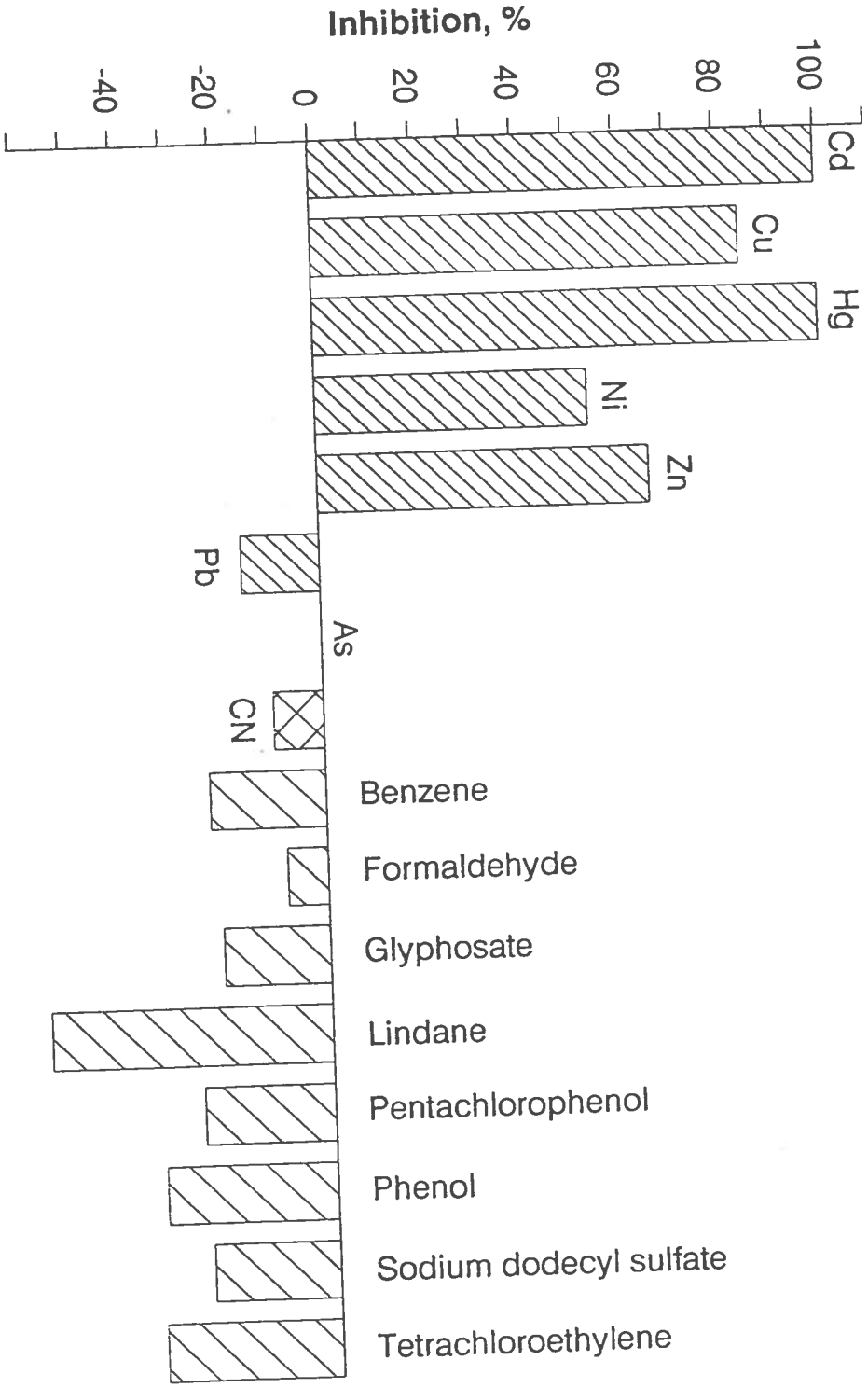

Fig 1.
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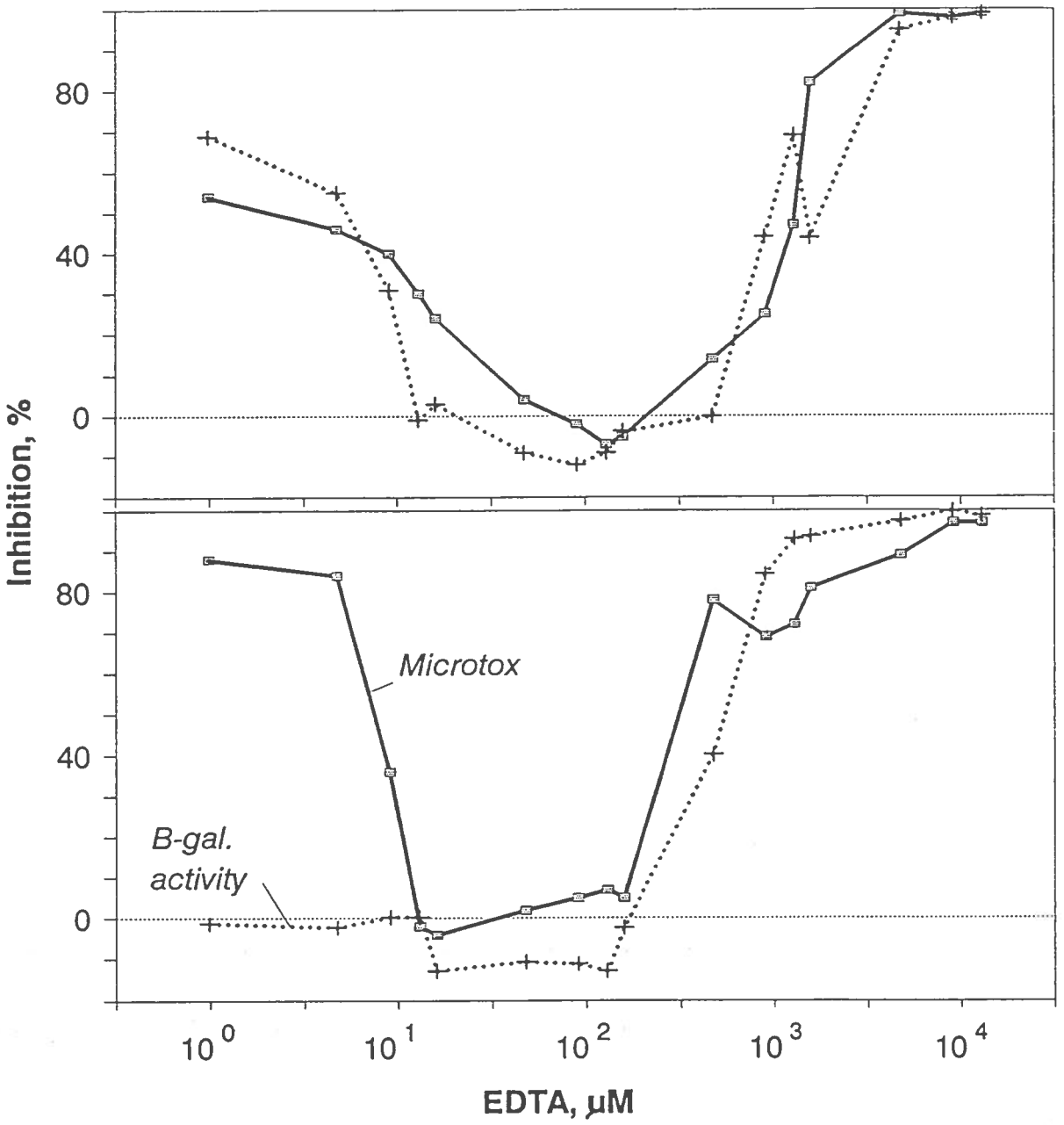

Fig 2
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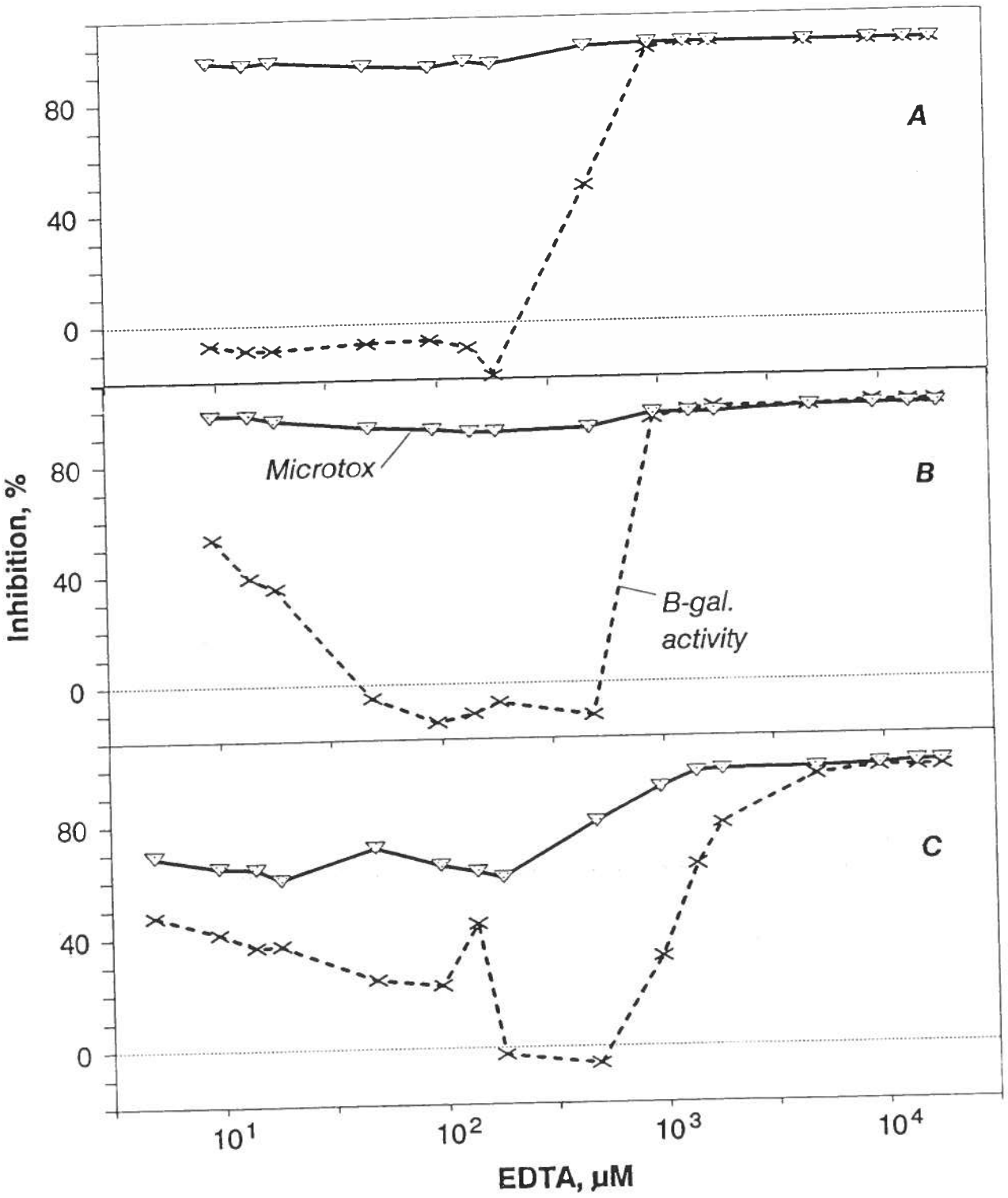

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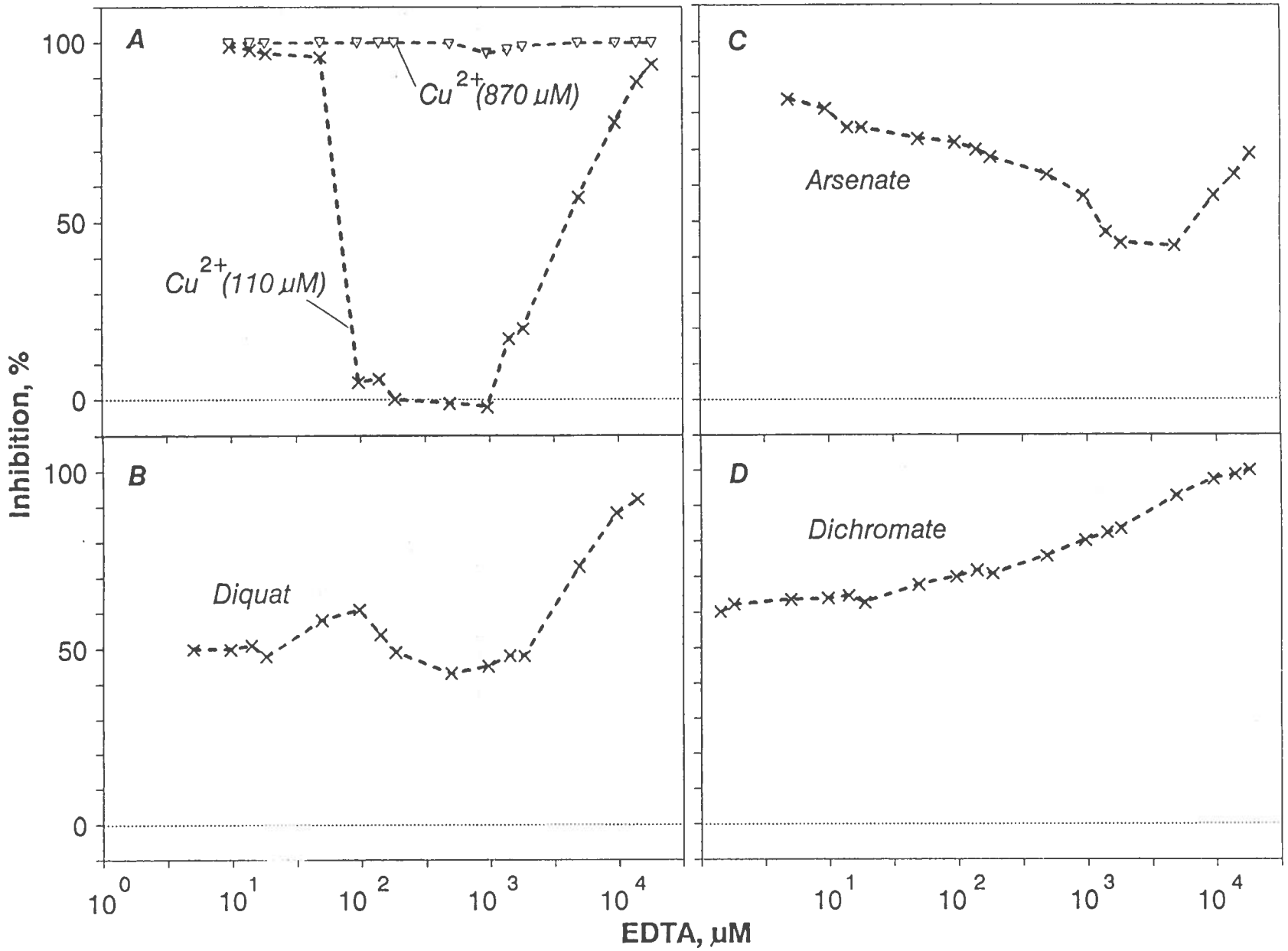

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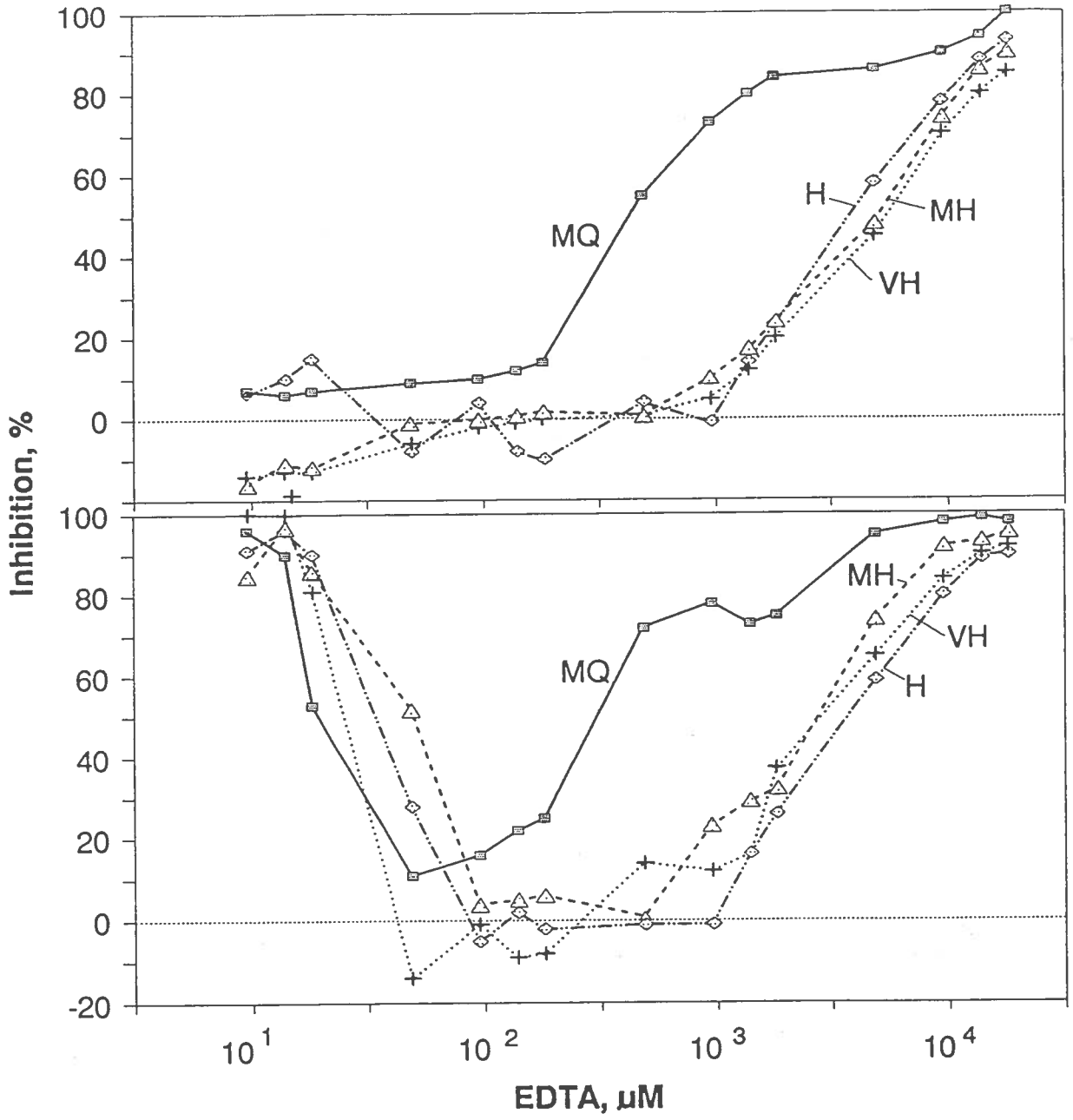

Fig 5
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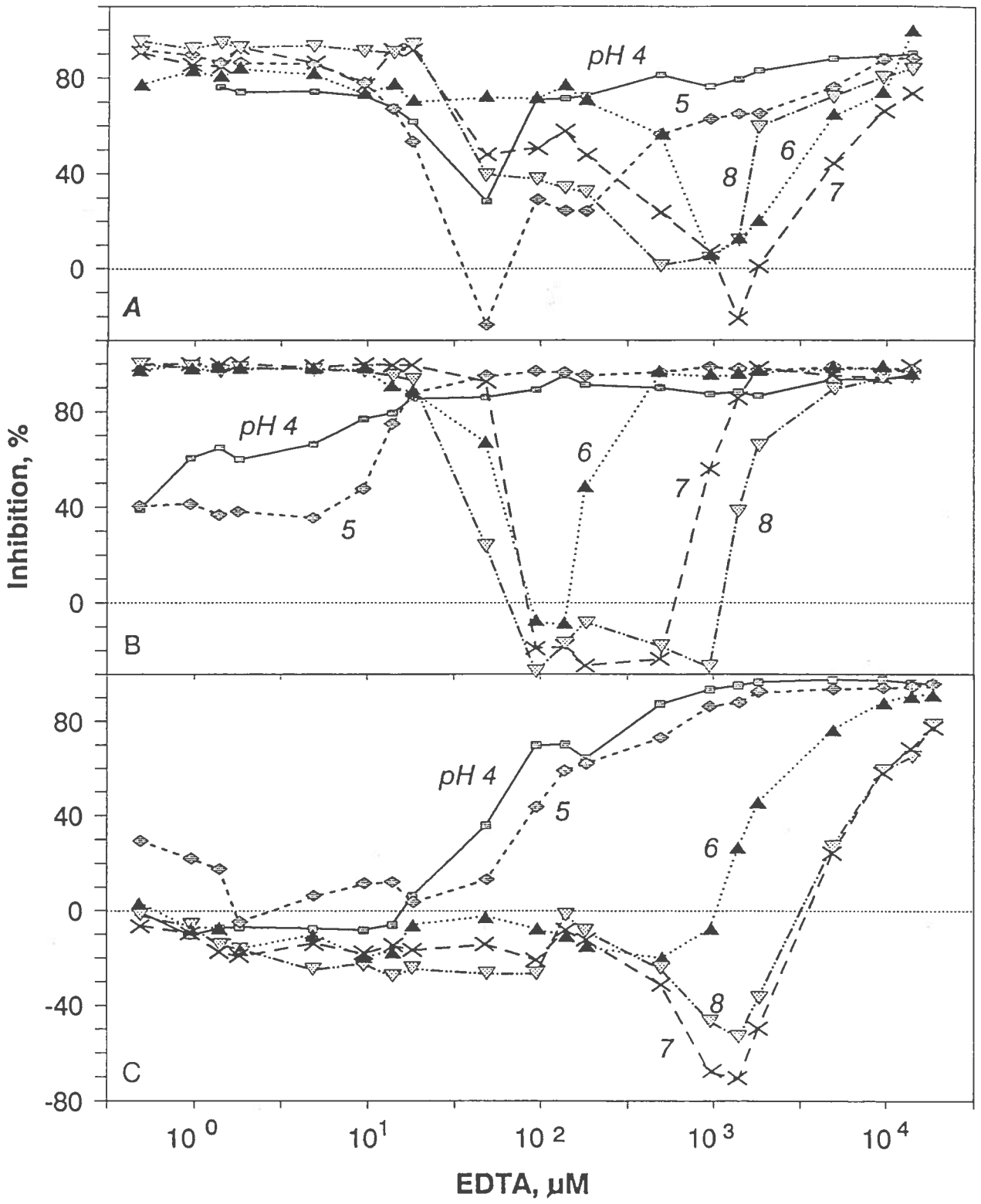

Fig 6
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