# SUSTAINED BACTERIAL REDUCTION of $Co^{III}EDTA^-$ in the PRESENCE of COMPETING GEOCHEMICAL OXIDATION DURING DYNAMIC FLOW

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## ABSTRACT

Radionuclides and metals can be mobilized by chelating agents typically present in low-level radioactive liquid wastes. $^{60}$Co-EDTA in the form $^{60}Co^{III}EDTA^-$ represents a highly stable (log $K_{Co(III)EDTA}$ = 43.9) and mobile form of this radionuclide. By contrast, the reduced form of this metal-ligand complex, $^{60}Co^{II}EDTA^{2-}$, is much less stable (log $K_{Co(II)EDTA}$ = 18.3) and less mobile. There is an increasing awareness that dissimilatory metal-reducing bacteria (DMRB) can be used to mediate redox transformations of metals and radionuclides whose stability and mobility are governed by their oxidation state. We conducted a series of column experiments to provide an improved understanding of $Co^{III}EDTA^-$ reduction by the facultative anaerobe *Shewanella alga* BrY (BrY). Experiments were conducted under growth conditions using lactate as carbon and energy source. We were able to demonstrate the sustained reduction of $Co^{III}EDTA^-$ in column flow experiments with the desired result that a less stable, less mobile product was formed. The amount of reduction varied directly with the fluid residence time in the columns. In the presence of a suitable mineral sorbent ($Fe(OH)_3$), Co-EDTA transport was delayed as a direct consequence of the bacterial reduction reaction. Even in the presence of a strong mineral oxidant ($\beta$-$MnO_2$) the net reduction of $Co^{III}EDTA^-$ dominated the fate and transport of this species. The system was stable after flow interrupts, and metal-reducing activity could be revived after flushing the columns for 5 days with nutrient-free solution. Furthermore, we demonstrated that BrY could grow and carry out sustained reduction using geochemically derived $Co^{III}EDTA^-$ as terminal electron acceptor. These results demonstrate that DMRB can be effective in the manipulation of redox-sensitive metals and radionuclides in a system characterized by the advective transport of solutes.

## INTRODUCTION

Scores of reports over the past several decades have documented the widespread problem of the migration of radionuclides away from waste disposal areas. Several mechanisms have been implicated as contributing to this problem including rapid transport through fractured geologic media in which some of the trenches are located (*1-3*), facilitated transport through sorption to mobile colloids (*4*), association with mobile natural and synthetic organic chelates (*5-10*), and oxidation-reduction reactions that promote the solubility, and thus the enhanced transport of the radionuclides (II, 12). In practice it seems likely that several of the above mechanisms act in concert to promote the migration of nuclides from their disposal sites.

A representative radionuclide of concern that has received considerable attention recently is cobalt-60 (""Co). In many situations, $^{60}Co$ has migrated farther than expected from disposal trenches and the enhanced mobility has been attributed, in part, to the formation of stable aqueous complexes with organic chelating agents such as ethylenediaminetetraacetic acid (EDTA). EDTA is a synthetic organic chelating agent that is used in cleaning and decontamination operations. EDTA increases the aqueous solubility of Co and alters its participation in other geochemical reactions. For example, EDTA stabilizes Co(III) in solution making the oxidation of Co(II) to Co(III) favorable by a broader array of minerals (12). The geochemical stability of Co-EDTA complexes, and thus the fate and transport of $^{60}Co$ in the subsurface, is strongly dependent on the oxidation state of Co ($Co^{2+} + EDTA^{4-} \rightleftharpoons Co^{II}EDTA^{2-}$, log $K_{Co(II)EDTA}$ = 18.3; log $K_{Co(III)EDTA}$ = 43.9). The oxidation of $Co^{II}EDTA^{2-}$ to $Co^{III}EDTA^{-}$ is catalyzed by Fe(III) oxides (12) and Mn(IV) oxides (II) common to the subsurface. The oxidized species ($Co^{III}EDTA^{-}$) is sorbed less strongly than the reduced species to soils and aquifer solids (12, 13) and complexed Co(III) is kinetically resistant to exchange reactions with other metals (14 - *17*) promoting the undesirable far-field transport of this radionuclide. Thus, the evaluation of geochemical factors that contribute to cobalt movement in soils and groundwater must include consideration of both aqueous complexation and redox reactions.

The geochemical processes governing nuclide mobility have received considerable attention and the potential impact that bacterial activity can have in determining the fate and transport of nuclides in the subsurface is gaining increasing attention. Two avenues of research have been the focus of the study into the microbiological effects on nuclide transport (1) bacterial degradation of organic chelators implicated in enhanced transport (18 - *24*), and (2) bacterially mediated oxidation-reduction reactions involving the radionuclide for the purpose of stabilizing the nuclide of concern with respect to cleanup objectives (e.g., either mobilizing the contaminant for site cleanup or immobilizing the nuclide to prevent plume migration)(*25* - 27).

The geochemical behavior, and thus the potential mobility in soils and groundwater, of many radionuclides is dictated by their oxidation state (e.g., Tc, U, Pu, Co) leading to the possibility of controlling their behavior by manipulating the oxidation state of the nuclide. A considerable body of work has demonstrated that dissimilatory metal-reducing bacteria can gain the energy for growth and reproduction by coupling the oxidation of $H_2$ or organic matter to the reduction of oxidized metals. Caccavo et al. (28) demonstrated that *Shewanella alga* BrY (BrY) can grow when supplied with $Co^{III}EDTA^{-}$ as sole terminal electron acceptor. More recently, Gorby et al. (2 *7*) extended these findings by coupling them with the observations of Jardine and Taylor (II) who reported the rapid and sustained oxidation of $Co^{II}EDTA^{2-}$ by the Mn(IV) oxide pyrolusite. Under no-growth conditions BrY was able to participate in a microbial-geochemical redox cycle in'which bacterially generated $Co^{II}EDTA^{2-}$ was reoxidized by pyrolusite to $Co^{III}EDTA^{-}$. However, the measured rate of geochemical oxidation was faster than the rate of microbial reduction suggesting that little net reduction of $Co^{III}EDTA^{-}$ would occur in the presence of mineral oxidants.

These initial studies (27, 28) provide an important first look into the coupled geochemical-microbiological system influencing the fate of $Co^{III}EDTA^-$. Nevertheless, groundwater environments differ from these batch studies in important ways that make it difficult to apply the results to groundwater systems. Aquifers are characterized by high solid to solution ratios that cannot be approximated in batch studies. To the extent that reaction rates and bacterial biomass may be expected to vary with surface area, this variable can have an important impact on experimental observations. Aquifers have a flow component that is not mimicked in batch studies. In flow-through systems, aqueous phase reactants and reaction products are constantly replenished to and removed from the system, respectively. Our objective was to study the reactive transport of $Co^{III}EDTA^-$ under the competing influences of microbial reduction and geochemical oxidation. Experiments were conducted under growth conditions by providing nutrients to promote the growth and reproduction of the bacterial inoculum.

## MATERIALS AND ANALYTICAL METHODS

Column experiments designed to investigate the biogeochemical processes governing the fate and transport of EDTA-complexed cobalt involved the use of the facultative anaerobe *Shewanella alga* BrY (29, 30). The solution injected to the columns had $Co^{III}EDTA^-$ as terminal electron acceptor and lactate was provided as the source of carbon and energy. Details of the experimental methods are provided below.

Growth and maintenance of bacteria: *Shewanella alga* strain BrY (BrY) was maintained on tryptic soy agar slants (30 g/L tryptic soy) and routinely cultured in tryptic soy broth (TSB; 30 g/L). Cells used in column experiments were grown aerobically in TSB at 28°C, harvested at mid to late log phase by centrifugation (5862 × g for 4 minutes at 4°C) and washed three times in 5 mM $CaCl_2$ /1 mM MOPS (3-[N-Morpholino]propanesulfonic acid), pH 7. After the final washing the cells were resuspended in 5 mM $CaCl_2$ / 1 mM MOPS to a final $OD_{590}$ = 2, which was verified to be $3 \times 10^9$ cells/mL by acridine orange direct count (AODC).

Column Experiments: We adopted column flow techniques to examine the influence of competing geochemical oxidation and microbiological reduction reactions on the reactive transport of $Co^{III}EDTA^-$ through packed sand columns. The solid phase used in the column was either silica sand, ferrihydrite ($Fe(OH)_3$)-coated sand, or pyrolusite ($\beta$-$MnO_2$)-coated sand. The results of a single experiment conducted under the relevant experimental conditions is presented in the figures. Preparation of the ferrihydrite-coated (1% Fe(III), w/w) and pyrolusite-coated (0.1% Mn(IV), w/w) sand has been detailed previously by Brooks et al. (12) and Jardine and Taylor (II), respectively. The surface loadings of Fe(III) and Mn(IV) were selected because they are characteristic of Fe(III) and Mn(IV) contents of aquifer solids at radioactive waste disposal areas (31, 32). Column packing and the flow experiments were conducted in an anaerobic glove box that was maintained under a positive pressure atmosphere of 95% $N_2$ / 5% $H_2$. The glove box was equipped with recirculating fan boxes fitted with Pd catalyst cartridges to remove trace amounts of oxygen. Samples were removed from the glovebox as soon as was practical. We verified that standard samples with BrY that were left in the glovebox for times

similar to the column effluent samples showed no change in $Co^{III}EDTA^-$ concentration. We further verified that there was no abiotic reduction of $Co^{III}EDTA^-$ by lactate or bacterial products by analyzing the same effluent samples over time and monitoring the lack of change in concentration of $Co^{III}EDTA^-$ and $Co^{II}EDTA^{2-}$.

Glass chromatography columns (Kontes, Vineland, NJ) were wet packed by filling the column with the washed bacterial suspension. The sand was saturated with an aliquot of the same suspension in a separate beaker and then the sand was transferred to the column in several lifts using a pipet. Physical properties of the columns are provided in Table 1. The columns were sealed and flushed for one to two hours with anaerobic reactant free carrier solution (5 mM $CaCl_2$ / 1 mM MOPS) to remove excess unattached bacteria and to remove any oxygenated solution from the column packing operation. Thus, when the injection of reactive tracers started, the number of bacteria in the column effluent was -$1 \times 10^6$ cells/ml. Ancillary batch experiments were conducted to examine the sorption of BrY cells to the sands under the conditions used to prepare the column packing suspensions. That is, 10 mL of bacterial suspension ($3 \times 1\ 0^9$ cells/mL in $CaCl_2$/MOPS) were contacted with 2 grams of sand. Samples collected over time indicated that bacterial sorption was complete within one hour. Suspensions of BrY that contacted the pure quartz, Mn-coated and Fe-coated sands lost 0%, 0%, and 43%, respectively, of the cells from suspension. This resulted in an initial cell concentration on the Fe-coated sand of about $1.5 \times 10^9$ cells/gram, prior to flushing the column. Preliminary column studies showed that cells of BrY were not retained in columns packed with the quartz sand. Therefore, to facilitate biomass retention, columns packed with the quartz or Mn-coated sand were mixed homogeneously with 10% (w/w) silica flour (99.88% pure, Timco Mfg.) and this mixture was used to pack the columns. Although a small fraction of the flour was actually packed in the columns, this was an effective method to physically retain some biomass in the columns.

An HPLC pump provided steady flow of either the carrier solution or the reactant solution ($Co^{III}EDTA^-$, lactate, plus bacterial nutrients, composition given below). Reactant solutions were kept separate and the influent was produced by using mixing valves in the HPLC pump. Prior to reaching the columns, solutions contacted only glass, Teflon, or PEEK (polyethylethylketone) surfaces. Reactant and carrier solutions were made anaerobic by vigorously sparging for 45 minutes per liter of solution with ultrapure He in glass bottles, then sealing the bottles and maintaining a positive He pressure (8 psi) on the solutions. Bromide breakthrough curves indicated that the columns were uniformly packed with negligible effects of dispersion (column Peclet number -200). Fluid residence times, calculated from the measured pore volume and flow rate, varied from 20 minutes to 140 minutes depending on the flow rate and column dimensions employed. During experiments in which the columns were packed with $Fe(OH)_3$ coated sand, the columns were wrapped in aluminum foil to prevent the potential photodegradation of $Fe^{III}EDTA^-$ (33) and the photoreduction of the $Fe(OH)_3$ solid phase (34). Samples of the column effluent were collected using an automated fraction collector.

Analytical Techniques: Bromide, $Co^{II}EDTA^{2-}$, and $Co^{III}EDTA^-$, were analyzed by ion chromatography using a minor modification of the method detailed by Taylor and Jardine (35). All of the EDTA in the influent was present as Co-EDTA, therefore, EDTA concentrations for mass balance calculations could be obtained by

summing the concentrations of $Co^{III}EDTA^-$ and $Co^{II}EDTA^{2-}$. Total aqueous cobalt, iron, and manganese were measured by atomic absorption spectrophotometry. Iron and manganese results presented in the paper have been corrected for the influent Fe and Mn. Subsamples for the determination of total protein and cell numbers were preserved with filtered (0.2 μm) 37% formaldehyde to yield a final formaldehyde concentration of 2% and stored at 4 °C for less than 10 days prior to analysis. Samples preserved in this manner were not used for the quantification of Co-EDTA species. Total protein was determined by Peterson's modification of the micro Lowry method (Sigma, St Louis, MO.). Cell numbers were determined by staining cells with 0.01% acridine orange and filtering onto a black polycarbonate filter (0.2 μm; Nuclepore); fluorescing cells were counted under ultraviolet light (36).

Control experiments for the sample preservation and handling method were conducted. Bovine serum albumin (BSA) standards were treated in a manner similar to that described above and compared to unpreserved BSA standards. In this way, we verified that this method did not influence the results of the protein determination. Time-course experiments using cells of BrY that were preserved as described demonstrated that there was no change in protein determination over the sample storage period. Similarly, there was no change in the cell numbers over time using this sample handling protocol, as determined by AODC.

Culture purity was verified for selected effluent samples by streaking on tryptic soy agar plates and growing the cells aerobically at 28°C. In addition, we compared Biolog® profiles of effluent samples to the column packing suspension and to pure cultures of BrY.

Reagents: $KCo^{III}EDTA \cdot 2H_2O$ was synthesized in our laboratory following the method of Dwyer et al. (1955). The column influent with reactive constituents (i.e., $Co^{III}EDTA^-$, bacterial nutrients) contained: 5 mM $CaCl_2$, 1 mM MOPS, 0.1 mM $KCo^{III}EDTA$, 0.05 mM $CaBr_2$, 0.005 g/L yeast extract, 0.134 mM KCl, 2.0 mM $Ca(lactate)_2$, 0.5 mM $NaH_2PO_4$, 2.5 mL/L trace mineral solution (7.85 mM $H_3NTA$, 24.9 mM $MgSO_4$, 3.3 1 mM $MnSO_4$, 17.1 mM NaCl, 0.658 mM $FeSO_4$, 0.901 mM $CaCl_2$, 0.954 mM $ZnSO_4$, 0.0627 mM $CuSO_4$, 0.0387 mM $AlK(SO4)_2$, 0.162 mM $H_3BO_3$, 0.121 mM $Na_2MoO_4$, 0.772 mM $NiCl_2$, pH adjusted to 6.5 with KOH). The final pH of the influent solution was 6.5. $CaCl_2$ was used as the ionic strength buffer for several reasons: to maintain similarity with the earlier work conducted on the geochemical oxidation of $Co^{II}EDTA^{2-}$ by metal oxides (II, 12); to promote the adsorption of cells to the sand surface using a divalent ion; to prevent the dispersion of colloidal fines associated with the metal-oxide coating which could occur if using Na' as the dominant cation.

## RESULTS AND DISCUSSION

We did not estimate the number of bacteria residing in the column during experiments although we did count the cells in effluent samples. The patterns observed indicate that the inoculum survived and grew. Cell numbers increased in the column effluent, usually by about one order of magnitude and remained there as long as nutrients were supplied (up to six weeks). This is consistent with the continual supply of cells from the column and inconsistent with a dead or dying inoculum that was washing out of the column. Decreasing cell numbers in

the effluent were only seen when nutrients were not provided in the influent, as described below. In addition, mass balance calculations demonstrated that the total number of cells eluted from the columns exceeded the total number of cells present in the packing suspension. As there were no cells in the influent, this means that there must have been a source term for bacteria in the column itself. These observations strongly suggest that the inoculum of BrY survived and grew during column experiments.

Under the conditions of dynamic *flow* used, *Shewanella alga* BrY was able to reduce $Co^{III}EDTA^{-}$ to $Co^{II}EDTA^{2-}$ continuously for the duration of the experiments conducted (up to six weeks). In experiments that were designed to study only the microbial reduction reaction the columns were packed with quartz sand. In a column packed with quartz sand, BrY was able to reduce $Co^{III}EDTA^{-}$ at a steady state amount of about 9% $Co^{III}EDTA^{-}$ reduced during a 19 minute residence time (Figure 1). The reduction of $Co^{III}EDTA^{-}$ was a bacterially mediated reaction as no $Co^{II}EDTA^{2-}$ production was observed in uninoculated columns (inset Figure 1) or in column experiments where no electron donor for BrY was supplied. Earlier work (*11,* 12) had shown that neither Co-EDTA species sorbed to the quartz sand and this was again confirmed as the breakthrough curves for total Co-EDTA and the nonreactive tracer bromide were coincident. The effluent pH for all column experiments remained steady at 6.5 - 6.8.

For all the column experiments conducted, there was an initial transient absence of bacterial metal-reducing activity. $Co^{III}EDTA^{-}$ was the first species detected in the column effluent followed by decreased concentrations accompanied by an equimolar increase in the concentration of $Co^{II}EDTA^{2-}$ (Figure 1). Based on a series of supplementary experiments (data not shown), we attributed the delayed onset of bacterial activity to the cell holding time required from cell harvesting to the initiation of the column experiment, which was typically a period of several hours. Nevertheless, BrY was able to carry out the sustained reduction of $Co^{III}EDTA^{-}$ in these experiments. In all cases we were able to demonstrate mass balance on total Co-EDTA, that is, BrY mediated a redox transformation of the cobalt moiety and did not consume EDTA.

In systems that contain Fe(III)-oxide coated sand, several reactions in addition to the microbial reduction of $Co^{III}EDTA^{-}$ must be considered to describe the system: the adsorption of $Co^{II}EDTA^{2-}$, and to a lesser extent $Co^{III}EDTA^{-}$ onto the Fe-coated sand, ligand-metal substitution reactions in which Fe(III) displaces Co(II) from EDTA (*37*), the bacterial reduction of solid phase Fe(III) (38, 40 - *42*), and the oxidation of $Co^{II}EDTA^{2-}$ to $Co^{III}EDTA^{-}$ by Fe(III)-oxide (12, 39). Overall, we observed more reduction of $Co^{III}EDTA^{-}$ in columns packed with Fe-coated sand than in columns packed with quartz sand (Figure 2; Table 1) for conditions of similar residence time. The greater extent of reduction in the presence of this mineral oxidant was likely the combination of several factors: the presumed increased biomass retention due to cell sorption to the Fe-coated sand, increased solute retention time due to sorptive retardation of the reactive species (*12*), and the gradual loss of the oxidative capacity of the Fe-oxide over time under anaerobic conditions (12). The occurrence of $Co^{III}EDTA^{-}$ concentrations above the influent value reflect the surface accumulation of this species and its subsequent displacement. Following the initial appearance of $Co^{III}EDTA^{-}$ there was no Co-EDTA detected for

almost 5 pore volumes (2.5 hours). This suggests 100% reduction of the $Co^{III}EDTA^-$ with the concomitant sorption and delayed transport of the more reactive reduced product. The release of the surface excess $Co^{II}EDTA^{2-}$ is evident in the breakthrough curve (Figure 2) as the concentration of this species temporarily exceeded the influent Co-EDTA concentration. After about 14 pore volumes (6.9 hours) the rate of $Co^{III}EDTA^-$ reduction decreased as indicated by the increased concentration of this species.

Total Fe eluted from the column, corrected for Fe in the influent, oscillated, averaging about 2 $\mu$M ± 0.278 (95% confidence interval). Iron concentrations present in the samples above a value expected for equilibrium between $CaCl_2$ and the solid phase (~ 0.7 $\mu$M) likely represents Fe that was liberated via simple complexation with constituents of the nutrient media (e.g., NTA, phosphate). We attempted to directly measure Fe(II) in the samples while they were still in the glovebox but chemical interferences with components in the nutrient media made the results unreliable. It is unlikely that either bacterial reduction of Fe(III) or the formation of $Fe^{III}EDTA^-$ contributed significantly to the Fe eluted from the column at this flow rate: the bacterial reduction of solid phase Fe(III) by BrY is not likely to be competitive in the presence of a more suitable soluble electron acceptor ($Co^{III}EDTA^-$)(*40* - 42); the formation of $Fe^{III}EDTA^-$ from $Co^{II}EDTA^{2-}$ is very slow (*41*), and we were able to demonstrate mass balance on total Co-EDTA. Nevertheless, because of the propensity for Fe(II) to readsorb to the Fe(III)-oxide surface, we concede that we may be underestimating the contribution of Fe(III) reduction in the system.

The influence of fluid residence time on the amount of reduction was tested using a flow interruption technique and performing another column experiment using longer residence time. The flow interruption technique was used on the current experiment. Flow through the column was stopped for 33.5 minutes (-1 pore volume) at pore volume -137. When flow was reinitiated, 100% of the $Co^{III}EDTA^-$ had been reduced to $Co^{II}EDTA^{2-}$ (inset, Figure 2). The concentration of $Co^{III}EDTA^-$ increased gradually over the next three pore volumes (-6 hours). There was no accompanying change in the concentration of Fe in the effluent samples, although this observation may change for flow interrupts significantly longer than one pore volume. These results demonstrate that the amount of reduction that was observed during flow was not limited by the amount of electron donor (lactate) available to BrY. In addition, subsequent manipulations where the nutrient concentration was increased up to 4 times, did not result in more reduction for the same hydrodynamic conditions. The results also show that the rate of $Co^{III}EDTA^-$ reduction is slower than the bulk fluid velocity through the column.

To explore further the effect of residence time, another column experiment with Fe-coated sand was conducted with a fluid residence time 4.7 times longer than the previous experiment. Following the transient breakthrough of $Co^{III}EDTA^-$, neither Co-EDTA species was eluted from the column for nearly 9 pore volumes (20 hours), indicating 100% reduction of $Co^{III}EDTA^-$ and the sorption and delayed transport of the more reactive species $Co^{II}EDTA^{2-}$. BrY reduced at least 97% of the influent $Co^{III}EDTA^-$ for more than the first 42 pore volumes (90 hours)(Figure 3). The slow increase in $Co^{II}EDTA^{2-}$ concentration following its initial rapid breakthrough is similar to earlier results where prolonged tailing of this species was observed (12). Within the

first 100 hours of this experiment, the number of cells in the column effluent increased from an initial value of 1 $\times 1\ 0^6$ cells/mL to a steady value of about $1.5 \times 10^7$ cells/mL for the duration of the experiment (data not shown). Subsequent to a 2.1 hour flow interrupt at pore volume -110 overall system performance improved: upon the restart of flow BrY was able to reduce 75% - 90% of the $Co^{III}EDTA^-$ for the next 20 days. The reason for the improved performance following the flow interrupt was not apparent from the effluent parameters that we monitored. There was no accompanying change in the number of cells/ml, cell protein content (0.0748 ± 0.0119 pg protein/cell), pH, or total Fe. The underlying cause for this observation merits further investigation.

During the first 50 pore volumes (116 hours) of this experiment substantial amounts of iron were released from the column. The period of high iron concentrations in the effluent coincided with the period where the sustained reduction of $Co^{III}EDTA^-$ exceeded 97%. In this instance, the increased iron likely was the result of the microbial reductive dissolution of the Fe(III)-oxide. Mass balance on Co-EDTA and the slow kinetics of $Fe^{III}EDTA^-$ formation make it unlikely that $Fe^{III}EDTA^-$ formation was significant. However, upon exposure to oxygen, the clear samples rapidly became clouded with a rust-colored colloidal suspension indicating that uncomplexed Fe(II) was being washed out of the column. The precipitation of colloidal Fe was not observed in samples from the column experiment with short residence time. After the first 50 pore volumes, the amount of Fe in the column effluent quickly decreased to less than 0.7 μM for the remainder of the experiment. The eventual decrease in Fe concentration likely reflects changes in the bacterial population that also led to less $Co^{III}EDTA^-$ reduction (Figures 2, 3) and changes in the Fe-oxide. For example, Roden and Zachara (38) demonstrated that during the reduction of solid phase Fe(III) by BrY, the adsorption of reaction products limited the rate and the extent of the reduction reaction.

As a final manipulation this column was flushed for 5 days with anaerobic 5 mM $CaCl_2$ / 1 mM MOPS, then the injection of $Co^{III}EDTA^-$ in nutrient solution was resumed. The metal-reducing activity of BrY was resuscitated quickly with relatively steady performance established within the first 24 hours and continuing for the next 10 days (Figure 4). Cell numbers in the column effluent, which had decreased during the flushing period from $2 \times 10^7$ to $2.5 \times 10^6$, quickly rebounded to $8 \times 1\ 0^6$. These results demonstrate the stability of the system and are similar to those of Caccavo et al. (28) who demonstrated that after long term (9 weeks) starvation, cells of BrY revived under aerobic growth regained metal-reducing activity.

Mn(IV) oxides are stronger oxidants and demonstrate the rapid and sustained oxidation of $Co^{II}EDTA^{2-}$ even under anaerobic conditions (II). Co-EDTA breakthrough curves from columns packed with pyrolusite (β-$MnO_2$)-coated sand exhibited aspects of the breakthrough curves from the columns packed with quartz sand and Fe-coated sand (Figure 5). Co-EDTA species do not sorb to this solid phase allowing the rapid transport of these constituents through the columns. However, a greater extent of $Co^{III}EDTA^-$ reduction was evident compared to the columns packed with quartz sand. Even in the presence of this strong mineral oxidant, BrY was able to reduce a substantial amount of $Co^{III}EDTA^-$ during the 23 minute residence time.

The importance of residence time was again evident from two intentional flow interrupts during which more $Co^{III}EDTA^-$ was reduced (Figure 5A). This again confirmed that the amount of reduction observed was not limited by the amount of available lactate. However, in the presence of the pyrolusite coated sand, note that following the reinitiation of flow, there was a transient increase in the concentration of $Co^{III}EDTA^-$ and Mn indicating that the bacterial reductant was depleted and no further reduction occurred while the geochemical reoxidation of bacterially generated $Co^{II}EDTA^{2-}$ proceeded (Figure 5A,B). This condition persisted for about 1 pore volume after the start of flow until fresh influent solution perfused the column and BrY had adequate available lactate again to begin reducing $Co^{III}EDTA^-$.

One possible explanation for the success of the microbial reduction reaction in the presence of this strong mineral oxidant is that the pyrolusite surface became inactivated from sorbed reaction products during the initial seven days of the experiment. Such a condition would result in little or no geochemical oxidation of $Co^{II}EDTA^{2-}$. The increased $Co^{III}EDTA^-$ concentration after the flow interrupts suggested that this was not the case. Nevertheless, we explored the possibility further by flushing the column of all Co-EDTA with anaerobic 5 mM $CaCl_2$ / 1 mM MOPS. An injection of anaerobic 0.09 mM $Co^{II}EDTA^{2-}$ in $CaCl_2$/MOPS without other nutrients was then initiated. The pyrolusite coated sand, which had already been in use for more than 450 pore volumes, demonstrated the rapid and sustained oxidation of $Co^{II}EDTA^{2-}$ to $Co^{III}EDTA^-$(Figure 6). A steady-state amount of oxidation of about 90% was rapidly achieved and maintained over 190 pore volumes (72 hours) when the experiment was terminated. Taking the residence time into consideration, this result is consistent with the earlier report of pyrolusite oxidation of $Co^{II}EDTA^{2-}$ (II).

In the column experiment where nutrients were injected, we were not able to distinguish the amount of reoxidation of bacterially generated $Co^{II}EDTA^{2-}$ versus $Co^{III}EDTA^-$ that had never been reduced but we did make an indirect estimate based on the amount of Mn released from the column. During the geochemical oxidation experiment described above, the steady state concentrations of $Co^{III}EDTA^-$ and Mn were 0.083 mM and 0.035 mM, respectively. This gives a ratio of about 2.44 millimoles $Co^{II}EDTA^{2-}$ oxidized per millimole Mn in the column effluent. The ratio slightly exceeds that expected when comparing equivalents, but this is not surprising given the complex Mn redox dynamics that have been proposed for this system (*11*) and partially confirmed (43, 44). The contribution of bacterial reduction of Mn(IV) to the total Mn in the effluent was assumed to be negligible for the experimental conditions given the slow measured kinetics of that reaction (27). The Mn breakthrough curve from the coupled biogeochemical column (Figure 5) indicates that (1) Mn concentrations increased following the flow interrupts (Figure 5B) which is consistent with the conjectured Co-EDTA dynamics during the same events, (2) between 28% and 100% of the $Co^{III}EDTA^-$ in the column effluent originated from the geochemical reoxidation of bacterially generated $Co^{II}EDTA^{2-}$, and (3) because Mn concentrations in the effluent were relatively steady (excluding the effects of flow interrupts) the primary reason for oscillations in the amount of $Co^{II}EDTA^{2-}$ was related to the bacterial reduction reaction, not the geochemical oxidation reaction.

An additional column packed with pyrolusite coated sand was run using a residence time that was 5.2 times longer than the previous experiment. BrY reduced more than 95% of the $Co^{III}EDTA^-$ for nearly 96 hours and, while exhibiting similar oscillations as seen previously, was able to reduce between 20% and 98% of the $Co^{III}EDTA^-$ for more than 28 days (data not shown). Bacterial cell numbers in the effluent rose to a steady state value of about $1 \times 10^7$ cells/ml. We again confirmed the ability of the pyrolusite coating to oxidize $Co^{II}EDTA^{2-}$ to $Co^{III}EDTA^-$ in the absence of bacterial activity, even after being in use for more than 330 pore volumes (28 days). Due to the increased residence time, 100% oxidation was sustained for more than 4 days, at which time this manipulation was terminated. During this geochemical oxidation trial, the ratio of millimoles $Co^{II}EDTA^{2-}$ oxidized per millimole Mn released was 2.38, which was in reasonable agreement with the ratio from the short residence time column.

As a final manipulation to this column, we flushed all Co-EDTA from the column, then began an injection of the bacterial nutrient solution but replaced $Co^{III}EDTA^-$ with 0.1 mM $Co^{II}EDTA^{2-}$. Thus, the sole electron acceptor (again assuming Mn(IV) reduction to be negligible) to support bacterial growth was $Co^{III}EDTA^-$ generated from the geochemical oxidation of $Co^{II}EDTA^{2-}$. Under these conditions, which had previously supported 100% oxidation of $Co^{II}EDTA^{2-}$, we observed less than 15% oxidation over a 1 O-day trial period (Figure 7A). Based on the amount of Mn eluted from the column, between 7 1% and 90% of the $Co^{II}EDTA^{2-}$ was being oxidized to $Co^{III}EDTA^-$. Over the same time period bacterial cell numbers, which had decreased to about $1 \times 1\ 0^6$ cells/mL during the antecedent manipulations, increased to about $7 \times 1\ 0^6$ cells/mL (Figure 7B). These results suggest that BrY was able to sustain growth on the geochemically produced electron acceptor $Co^{III}EDTA^-$ under these dynamic flow conditions.

As mentioned above, we concede that we may have underestimated the role of bacterial Fe(III) reduction in these experiments due to the readsorption of Fe(II) to the solid phase surface. This in turn may lead us to overestimate the role of bacterial $Co^{III}EDTA^-$ reduction and underestimate the role of the abiotic reduction of $Co^{III}EDTA^-$ via reduced iron. The products of bacterial Fe(III) reduction include solid phases such as siderite ($FeCO_3$), vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) or magnetite ($Fe_3O_4$), and aqueous Fe(II). The culture conditions when siderite is generated are described as 30 mM $NaHCO_3$ and 20% CO, headspace (*45,46*)- conditions that are not relevant to our experiments. Biogenic formation of vivianite has been observed when phosphate concentrations are 4 mM (*46*), eight times higher than the concentration used in these experiments. We are unaware of open literature reports that demonstrate that *Shewanella alga* BrY can produce magnetite, but even if it could, thermodynamic calculations for the experimental conditions indicate that magnetite cannot reduce $Co^{III}EDTA^-$ to $Co^{II}EDTA^{2-}$. By contrast, aqueous Fe(II) could reduce $Co^{III}EDTA^-$ to $Co^{II}EDTA^{2-}$ at the relevant experimental conditions. Nevertheless, if the abiotic reduction of $Co^{III}EDTA^-$ was occurring to a significant extent in the column experiments, one would expect that there would be more $Co^{III}EDTA^-$ reduction in the columns packed with Fe(III)-coated sand (potential for abiotic reduction, no appreciable geochemical oxidation) than in the columns packed with $MnO_2$-coated sand (no potential for abiotic reduction, demonstrated rapid and sustained

geochemical oxidation). In fact, the amount of $Co^{III}EDTA^-$ reduction in the two systems is comparable under similar hydrodynamic conditions (Table 1). These results suggest to us that the abiotic reduction reaction of $Co^{III}EDTA^-$ by aqueous Fe(II) is not a dominant process under these conditions.

Similarly, we may have underestimated the role of bacterial reduction of Mn(IV), leading to the formation of solid phase reduced Mn products in the column leading to the abiotic reduction of $Co^{III}EDTA^-$ by these products. We can find no published data nor a thermodynamic basis to validate these arguments. Formation of Mn-carbonate solids is highly unlikely at the CO, levels in these experiments, so we do not consider them further. Thermodynamic calculations indicate that the reduction of $Co^{III}EDTA^-$ by all likely Mn(III) and Mn(II) phases is thermodynamically prohibited at the column conditions. $Co^{III}EDTA^-$ reduction by Mn(II) at pH 6.5 first becomes feasible when the expressed concentration of Mn(II) exceeds 3.42 Molar, which is greater than the total Mn concentration in the columns by a factor of more than 120. Thus, the abiotic reduction of $Co^{III}EDTA^-$ by Mn(III) or Mn(II) products is not possible in our experiments.

Both field and laboratory investigations have demonstrated that the interaction of $Co^{II}EDTA^{2-}$ with common soil and aquifer minerals results in the oxidation of this species to the highly stable and mobile $Co^{III}EDTA^-$. We were able to demonstrate the sustained bacterial reduction of $Co^{III}EDTA^-$ in column flow experiments with the desired result that a less stable, less mobile product was formed. The amount of reduction varied directly with the fluid residence time in the columns suggesting that the reduction process is time-dependent on the order of minutes to hours. In the presence of a suitable mineral sorbent ($Fe(OH)_3$), Co-EDTA transport was delayed as a direct consequence of the bacterial reduction reaction. Even in the presence of a strong mineral oxidant ($MnO_2$) the net reduction of $Co^{III}EDTA^-$ dominated the fate and transport of this species. Under the experimental conditions employed, the system was stable after flow interrupts, and metal-reducing activity could be revived after several days of flushing with nutrient-free solution. Furthermore, we demonstrated that BrY could grow and carry out sustained reduction using geochemically derived $Co^{III}EDTA^-$ as terminal electron acceptor. These results demonstrate that dissimilatory metal-reducing bacteria can be effective in the manipulation of redox-sensitive metals and radionuclides. In addition, these results are relevant to active biowall technologies that could be used to immobilize subsurface contaminants from migrating groundwater plumes. Sustained contaminant reduction is essential for their long-term success, and the current study offers some of the first evidence that this technology is plausible for immobilizing redox sensitive metals and radionuclides.

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Table 1: Selected properties of the columns used in experiments.

| Solid Phase | dimensions (diam × length, cm) | flow rate ($cm^3$/min) | bulk density ($g/cm^3$) | saturated water content ($cm^3/cm^3$) | fluid residence time (minutes) | Experiment | % $Co^{III}EDTA^-$ reduced † | Figure # |
|---|---|---|---|---|---|---|---|---|
| Quartz sand | 1 × 15.5 | 0.05 | 1.43 | 0.50 | 122 | uninoculated control | 0 | inset Fig. 1 |
| | 1 × 14.45 | 0.1963 | 1.65 | 0.34 | 19 | inoculated, nutrient addition | 8.7 | 1 |
| $Fe(OH)_3$-coated sand | 1 × 14.05 | 0.1966 | 1.24 | 0.54 | 29 | inoculated, nutrient addition | 67.5 | 2 |
| | 2.54 × 10.75 | 0.2012 | 1.34 | 0.52 | 139 | inoculated, nutrient addition | 77.5 | 3 |
| | | same as the parameters in the row above | | | | reviving inoculum after 5-day flush with $CaCl_2$ | 67.8 | 4 |
| $MnO_2$-coated sand | 1 × 13.45 | 0.1998 | 1.46 | 0.45 | 23 | inoculated, nutrient addition | 72.4 | 5 |
| | | same as the parameters in the row above | | | | geochemical oxidation of $Co^{II}EDTA^{2-}$ | 92.2 ‡ | 6 |
| | 2.54 × 10.95 | 0.1994 | 1.52 | 0.44 | 121 | inoculated, nutrient addition | 71.5 | not shown |
| | | same as the parameters in the row above | | | | biogeochemical cycling of $Co^{II}EDTA^{2-}$ | 61.4 - 80.4 § | 7 |

† The percent $Co^{III}EDTA^-$ reduced was calculated by integrating the area under each breakthrough curve using the trapezoidal rule and is expressed as a percentage of the total $Co^{III}EDTA^-$ introduced to the column.

‡ This represents the percentage of $Co^{II}EDTA^{2-}$ that was oxidized to $Co^{III}EDTA^-$ by contact with the pyrolusite surface.

§ Amount of $Co^{III}EDTA^-$ reduced calculated as total $Co^{II}EDTA^{2-}$ oxidized to $Co^{III}EDTA^-$ (estimated from Mn eluted from the column) minus the amount of $Co^{III}EDTA^-$ eluted from the column.

**List of Figure Captions**

Figure 1: Observed solute breakthrough curve (relative concentration versus dimensionless time expressed as pore volumes) for a column packed with quartz sand and inoculated with *Shewanella alga* BrY. The relative concentration is calculated as = measured sample concentration, C, divided by the solute concentration in the influent, C,. Note that the upper x-axis represents real time. The injection solution consisted of 0.1 mM $Co^{III}EDTA^{-}$, 2.0 mM $Ca(lactate)_2$, 0.05 mM $CaBr_2$, plus bacterial nutrients in 5 mM $CaCl_2$. Fluid residence time for this column, determined from the measured pore volume and flow rate was 19 minutes. Inset: results from an uninoculated control column showing no abiotic reduction of $Co^{III}EDTA^{-}$, even with prolonged residence time (Table 1).

Figure 2: Breakthrough curve for Co-EDTA species from column packed with $Fe(OH)_3$-coated $SiO_2$. Injection conditions were similar to those listed in the caption to Figure 1; fluid residence time was 29 minutes. The arrow marks when flow was intentionally stopped for 33.5 minutes. Inset: Detailed presentation of the events surrounding the flow interrupt.

Figure 3: Breakthrough curve for Co-EDTA species from a column packed with $Fe(OH)_3$-coated $SiO_2$, using a longer fluid residence time = 139 minutes. Flow was intentionally stopped for the indicated amount of time at the position marked by the arrow.

Figure 4: Revival of $Co^{III}EDTA^{-}$ reduction by BrY following a five day period of flushing the column with anaerobic 5 mM $CaCl_2$. These data were collected as one manipulation of the same column depicted in Figure 3. $Co^{III}EDTA^{-}$ reduction began almost immediately upon injection and continued over the next 10 days.

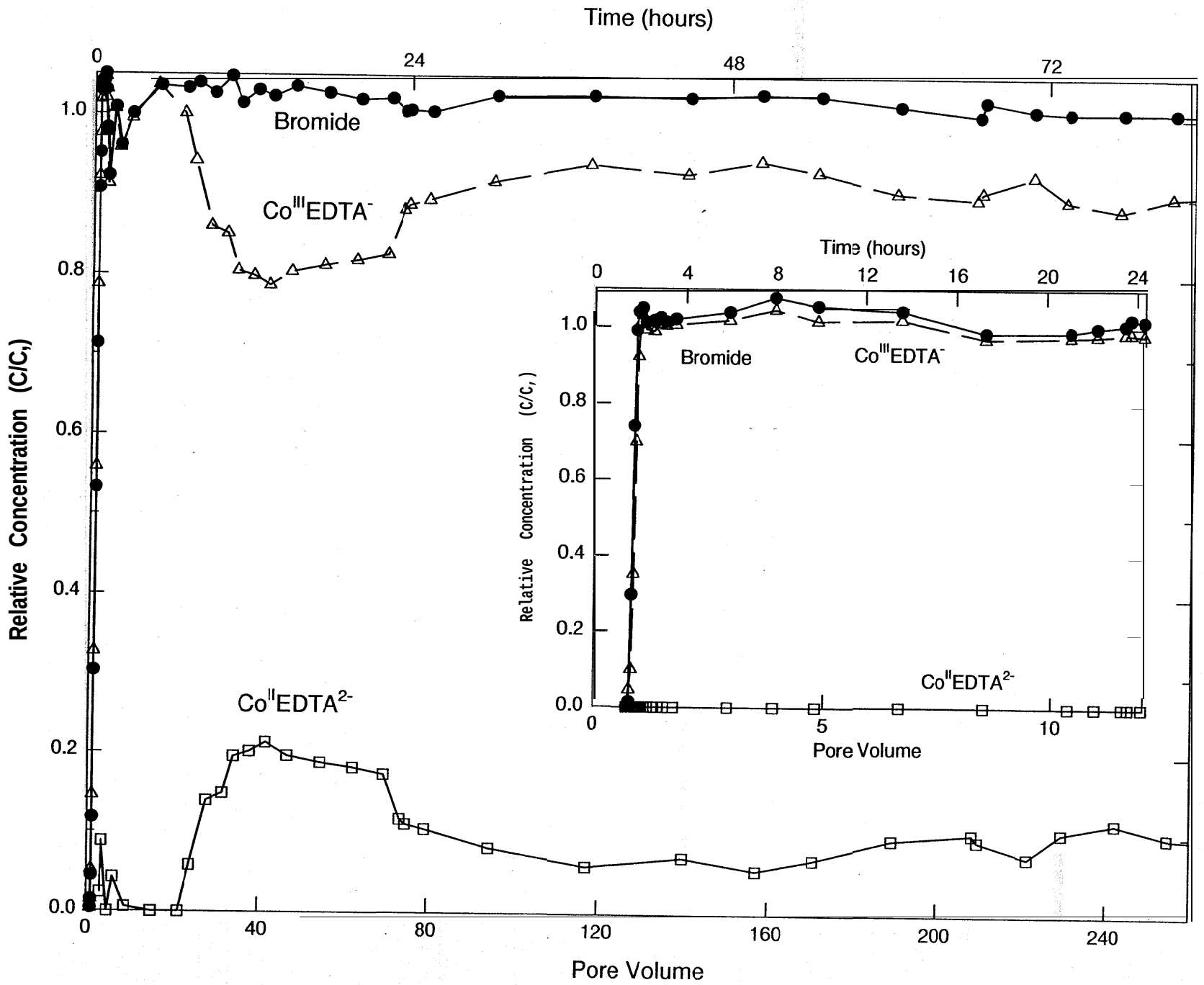
Figure 5 : Breakthrough curve for Co-EDTA species (A) and Mn (B) from a column packed with $MnO_2$-coated $SiO_2$ and inoculated with BrY, fluid residence time = 23 minutes. BrY was able to effect the net reduction of $Co^{III}EDTA^{-}$ even in the presence of this strong mineral oxidant. During the flow interrupts, BrY continued to reduce $Co^{III}EDTA^{-}$ until available electron donor was depleted, although the geochemical oxidation of microbially generated $Co^{II}EDTA^{2-}$ continued. Mn concentrations have been corrected for Mn in the influent.

Figure 6: Oxidation of anaerobic 0.09 mM $Co^{II}EDTA^{2-}$ by $MnO_2$-coated $SiO_2$ using the same column represented in Figure 5. The pyrolusite showed the rapid and sustained oxidation of $Co^{II}EDTA^{2-}$ even after being used in other manipulations for more than 450 pore volumes (175 hours).

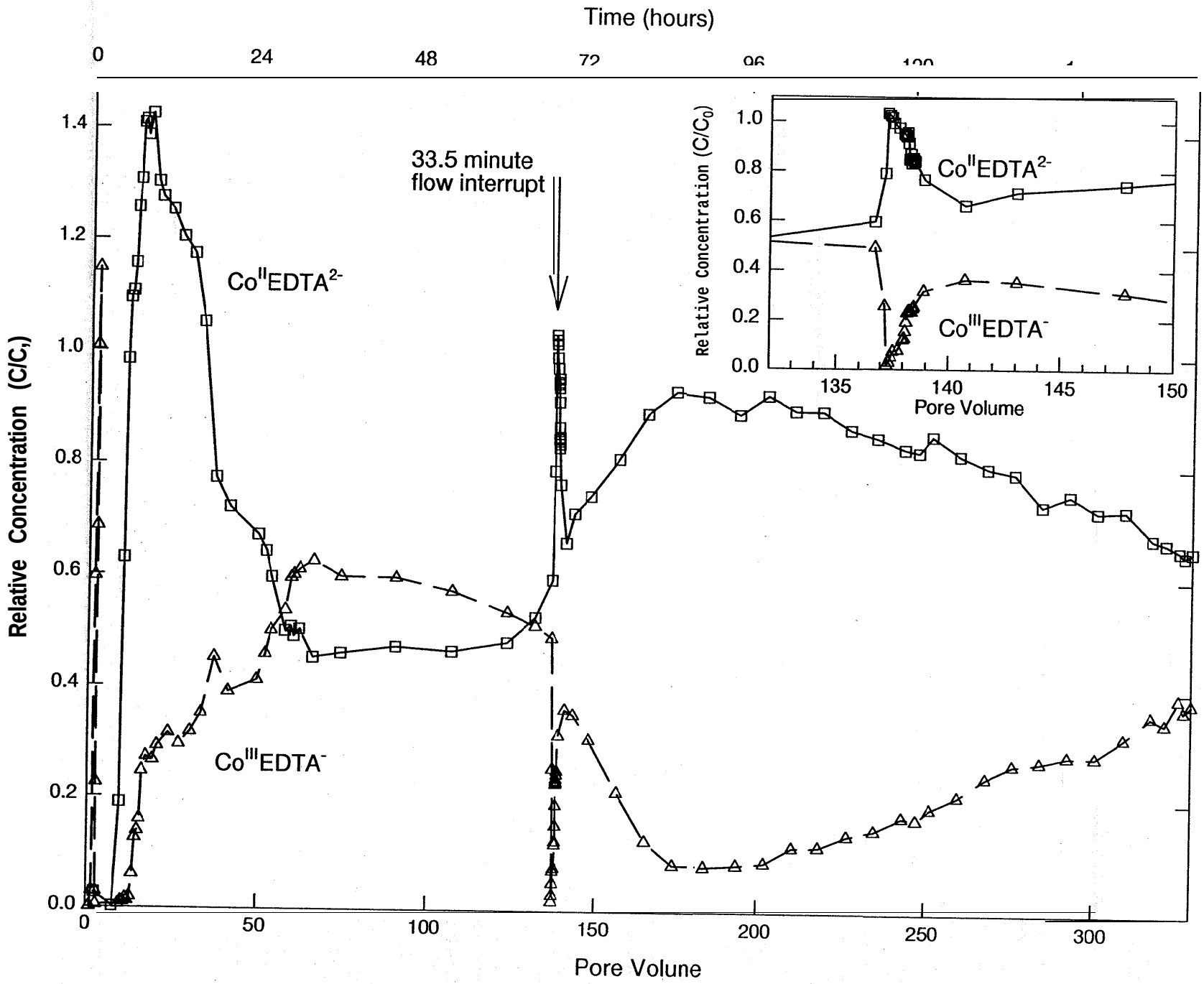
Figure 7: Breakthrough curve for Co-EDTA species and Mn (A) from a column packed with $MnO_2$-coated $SiO_2$ and inoculated with BrY, fluid residence time = 12 1 minutes. Mn concentrations have been corrected for Mn in

the influent. This experiment was started after using the column for other manipulations for more than 400 pore volumes (33 days). Prior to starting the experiment shown, we verified the ability of the $MnO_2$ to oxidize 100% of $Co^{II}EDTA^{2-}$. Injection solution consisted of 2.0 mM $Ca(lactate)_2$ as electron donor and energy source for BrY, 0.1 mM $Co^{II}EDTA^{2-}$ plus the other bacterial nutrients. The sole electron acceptor available for BrY was geochemically produced $Co^{III}EDTA^{-}$. Cell numbers in the column effluent increased in response to this treatment (B) indicating that BrY was able to grow and reproduce under these experimental conditions.

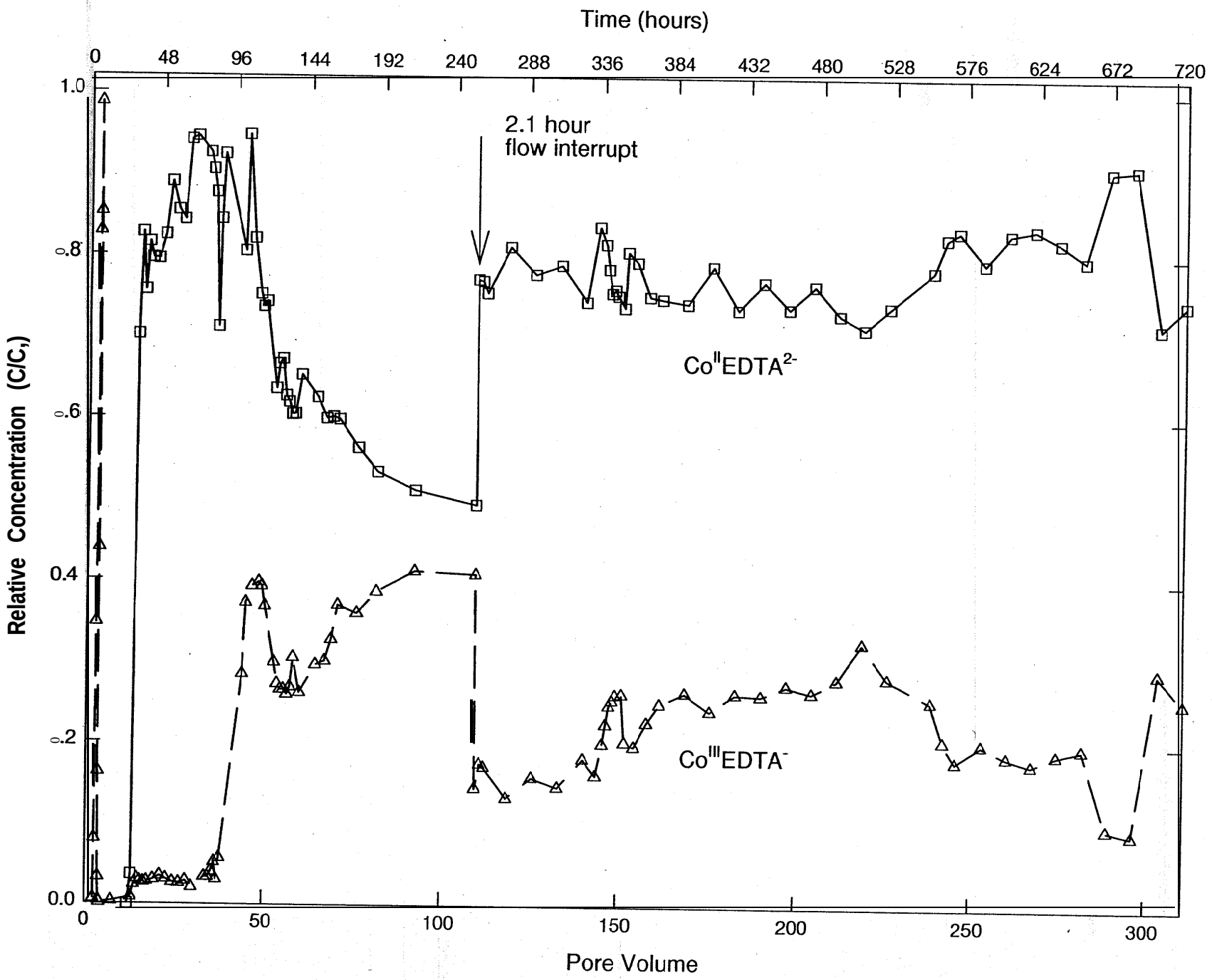
Brooks et al., Figure 1



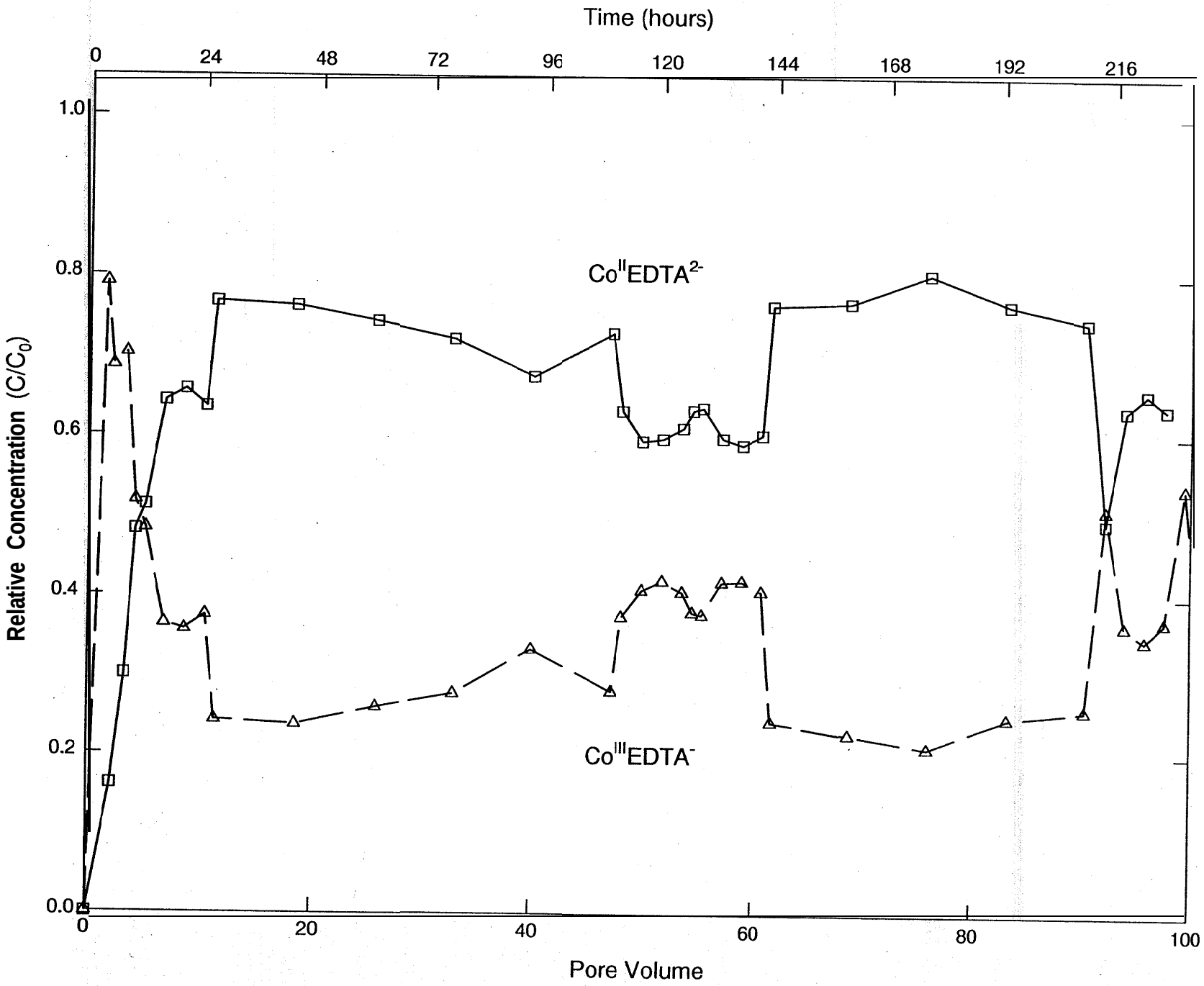
Brooks et al., Figure 2



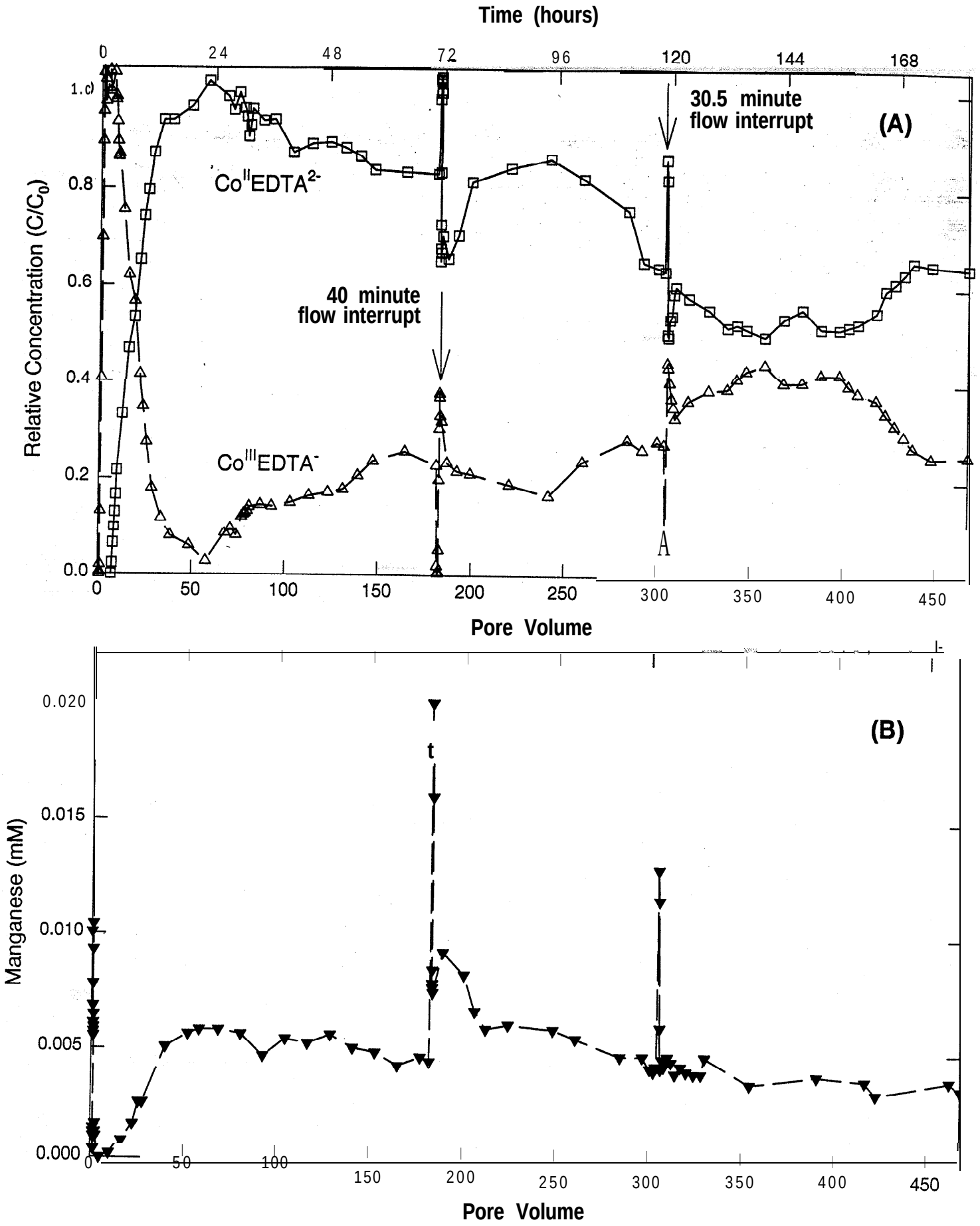
Brooks et al., Figure 3



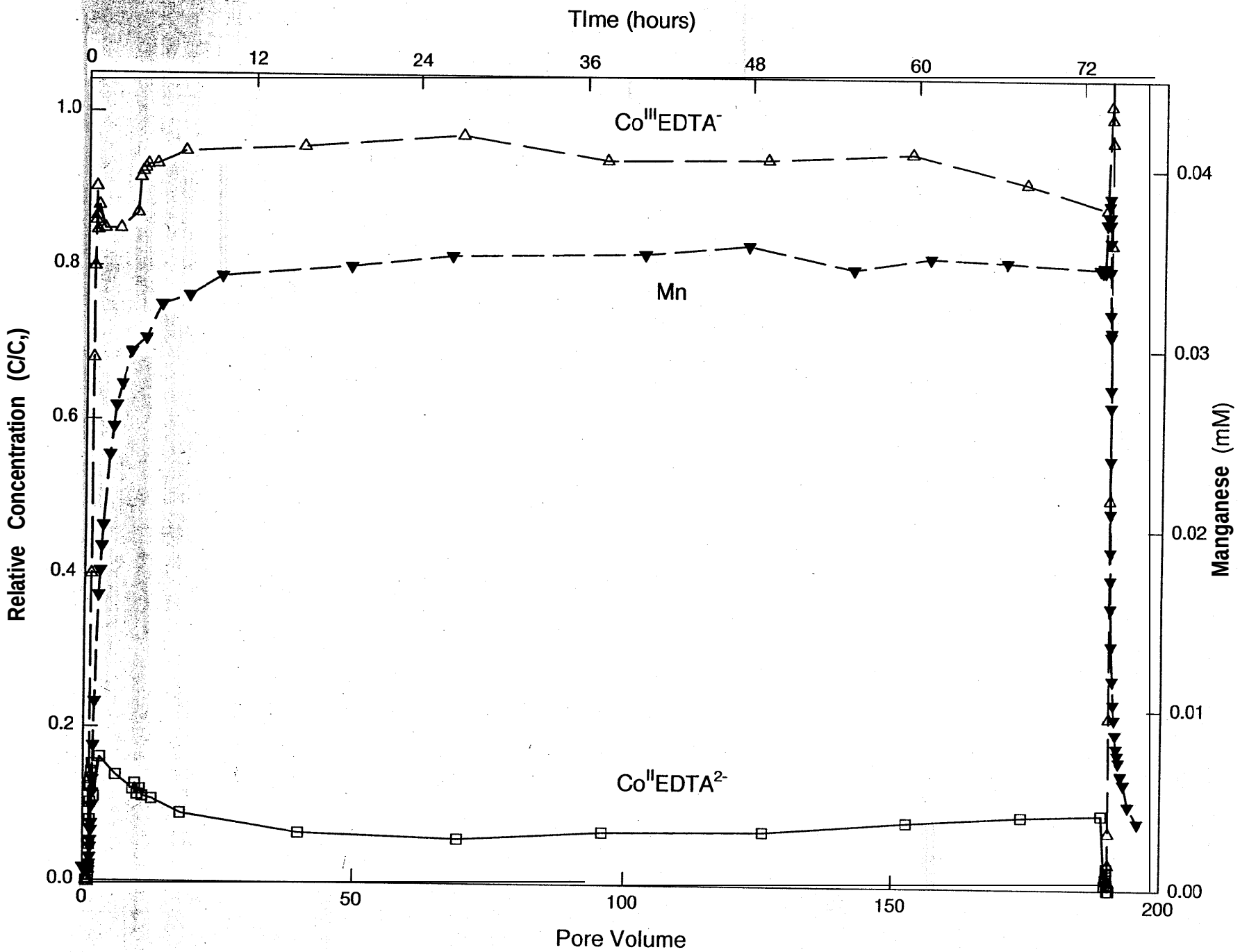
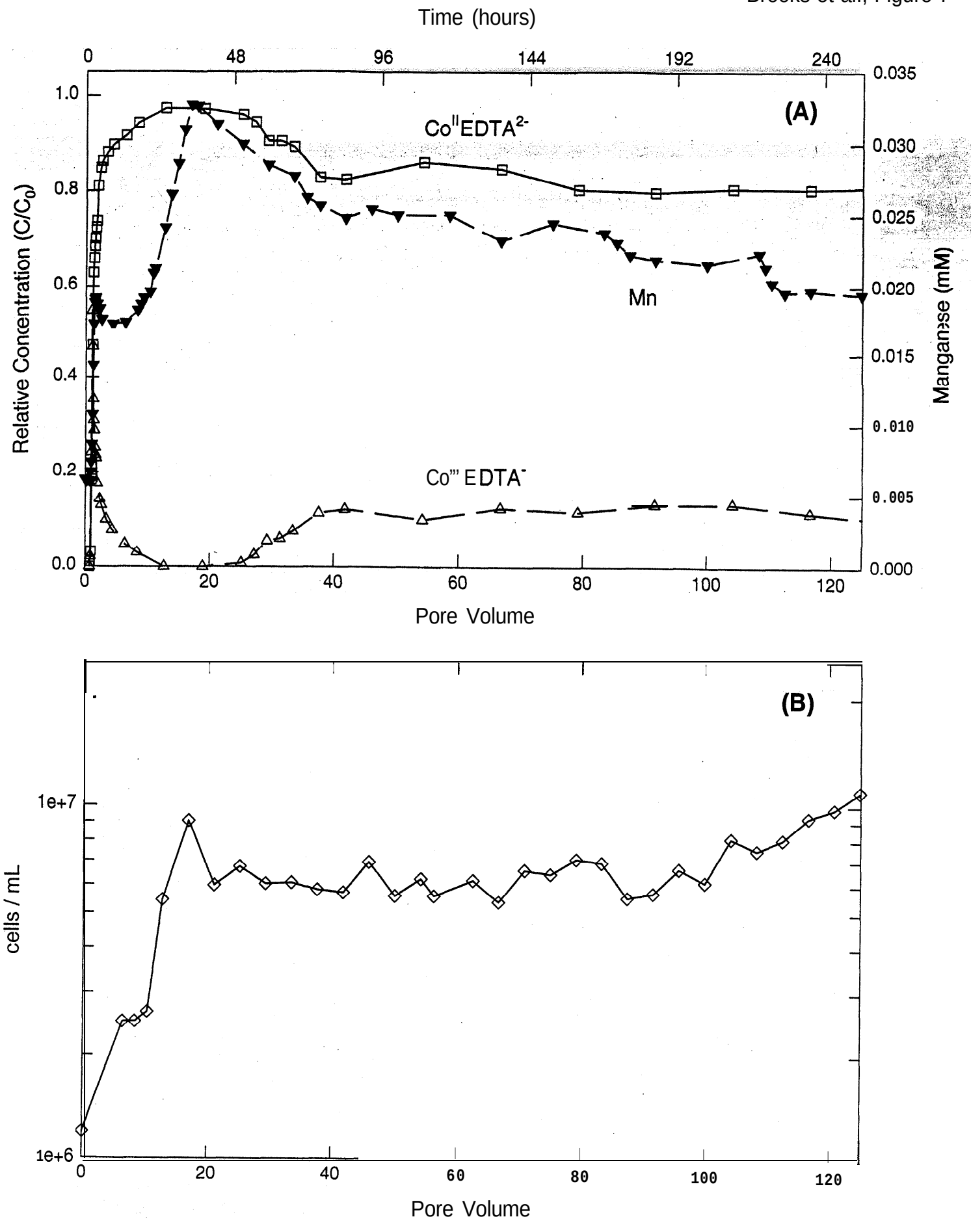
Brooks et al., Figure 4



Brooks et al., Figure 5



Brooks et al.Figure 6



Brooks et al., Figure 7