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Mass Balance of Heavy Metal Uptake by Encapsulated Cultures of *Klebsiella aerogenes*

Thomasine Rudd, Robert M. Sterritt, and John N. Lester

Public Health Engineering Laboratory, Imperial College, London, England

Abstract. Dialysis was employed as a method of speciating heavy metals in cultures of an extracellular polymer forming strain of *Klebsiella aerogenes*. A noncapsulated strain of the same bacterium was used as a control, and a mass balance of copper, cadmium, cobalt, nickel, and manganese in batch culture at pH 4.5 and pH 6.8 and in continuous culture at pH 6.8 was constructed. Copper and cadmium were accumulated by the cell during rapid proliferation whereas all 5 metals were bound nonspecifically by extracellular polymer produced during stationary phase and at low dilution rates. The presence of extracellular polymer appeared to inhibit cellular uptake of nickel. At the lower pH, metal uptake was considerably reduced. The results are discussed in the context of metal removal in the activated sludge process of waste water treatment.

Introduction

Industrial and domestic water use results in the discharge of heavy metals in sewage effluents to natural aquatic systems. Their potential environmental impact may be increased as a result of a greater trend towards water reuse [16]. Investigations have been made into the toxic effects of such heavy metals on the growth and activity of aquatic bacteria, both in pure culture [19, 26] and in mixed populations [1, 15, 18].

Bacteria have been shown to adapt to growth-inhibiting concentrations of metal ions [19]. A frequent response is an extension of the lag phase prior to the onset of growth, the length of the lag phase varying with the concentration of the metal [10, 22]. Once acclimated, bacterial cells are capable of accumulating metal, resulting in its incorporation in food chains and transfer to higher organisms by biomagnification [21].

Metal ions are accumulated intracellularly by active, energy-expending transport into the cell [8]. Extracellular uptake occurs by ion exchange with the polyanions of the cell wall. Substantial metal binding to the cells walls of gram-positive bacteria and to a lesser extent gram-negative bacteria has been demonstrated [2]. Additional extracellular uptake occurs in capsulated strains of bacteria by complexation with extracellular polymers [4].

Extracellular polymers exist as discrete capsules or dispersed slime and can bind large quantities of water and cations to optimize the cell's local environ-

ment [11]. The binding of heavy metals by polymer has been demonstrated both *in vivo* [6, 10, 12, 13] and with polymer extracted from several species of bacteria [3, 5, 10] and Rudd et al. (in press). This ability is of great importance in the removal of heavy metals during biological waste water treatment, as it enhances the uptake of predominantly soluble metals which are poorly removed by other mechanisms [4]. The efficiency of metal removal by extracellular polymers depends on the partition of metals between the solid and dissolved phases; production of soluble forms of polymer under certain conditions [24] would tend to maintain metal solubility and thus adversely affect removal.

Metal uptake by both the cells and extracellular polymer of bacteria can be affected by environmental factors, such as the chemical constituents of a particular aquatic system, by the occurrence of synergistic or antagonistic reactions among the elements present [14]. Other factors such as pH, E_h, temperature, and hydrostatic pressure can influence both the biochemical activity of bacteria and the speciation of metals, and consequently any interactions occurring between them [28].

This study has investigated the distribution of metals taken up by a capsulated bacterium among the sites of accumulation available, and the effect of some cultural conditions on the mass balance of metals within the culture. It was designed to elucidate the mechanisms involved in metal removal during biological waste water treatment.

Materials and Methods

Bacterial Cultures

An organism representative of the largely gram-negative bacterial population of activated sludge and capable of copious high molecular weight extracellular polymer production was chosen for experimental studies [4, 9].

Two pure cultures of *K. aerogenes,* a polymer-forming capsulated strain, NCTC 8172, and a noncapsulated strain, NCTC 9528, were grown in batch culture and in continuous culture as previously described [24]. A defined medium containing the following A.R. grade components (g l^{-1}): sucrose, 10; $(NH_4)_2 SO_4$, 0.3; K_2SO_4, 1; NaCl, 1; $MgSO_4\ 7H_2O$, 0.2; $CaCl_2\ 6H_2O$, 0.02; $FeSO_4\ 7H_2O$, 0.001 was buffered to pH 6.8 by the inclusion of (g l^{-1}) $NaH_2PO_4\ 2H_2O$, 2; $K_2HPO_4\ 3H_2O$, 2. Sterile metal stock solution containing 10^{-2} *M* copper, cadmium, cobalt, nickel, thallium (as nitrate salts), and manganese (as the sulphate salt) was added aseptically to the basal medium after sterilization in order to minimize precipitation. Triplicate batch shake cultures were made of both strains, two containing 10^{-5} *M* metals and one metal free control. There was no pH control in these cultures apart from the buffering capacity of the medium, which had an initial pH of 6.8, therefore some batch cultures were also grown in the chemostat which was equipped for pH control. Batch and continuous cultures of both strains in the chemostat were maintained at pH 6.8. Culture samples for viable count were serially diluted in 0.1 M phosphate buffer, plated onto casitone-glycerol-yeast extract agar [23] and incubated at 25°C for 24 h.

Sampling

The viable count of the batch, shake cultures was determined at the end of log phase and samples of 10 ml were taken for analysis after 6 days growth. Single samples of 25 ml (< 1% culture volume)

were taken from the chemostat batch cultures every 2–4 h during log phase and then daily for 6 days. In this case, no replicate samples were taken so as to minimize the cumulative loss of culture volume from the vessel. Triplicate 25 ml samples were taken at each dilution rate during continuous culture. Viable counts and polymer and metal determinations were carried out on all samples taken from the chemostat. An equilibrium period equivalent to 3 volume changes was allowed between sampling at each dilution rate. Half of each sample taken from the cultures was filtered through a 0.2 μm micropore filter. Those for metal analysis were acidified to contain 1% nitric acid and sealed. All samples were stored at 4°C.

Metal Speciation

The medium was left to stand for 3 h after metal addition in order to allow any precipitates to settle. The concentration of soluble metal was then determined in the supernatant. Precipitation in the batch shake cultures, in which the pH dropped from 6.8–4.5, was estimated by adding metals to filtrate from the 6-day-old metal-free culture, and the concentration of soluble metal remaining after refiltration was determined. This was done in order to assess the effect of precipitation in the spent culture medium after the reduction in pH had occurred. Metal uptake by the cultures was determined by dialysis of samples in visking tubing with a molecular weight cut-off of 12,000–14,000 (Medicell Int., London) for 70 h at 4°C. The molecular weight of this capsular serotype, K64, of *K. aerogenes* extracellular polysaccharide has previously been specified as 1.7×10^6 [9]. Use of this size dialysis tubing therefore permitted a separation of metal bound by cells and extracellular polymer from free metal and any lower molecular weight complexes derived from bacterial metabolites.

A 0.1% solution of $MgSO_4$, a constituent of the culture medium, with the pH adjusted to 6.8 was used as the dialysis medium in order to reduce the osmotic shock to cells and to neutralize any charge on the dialysis membrane. *In situ* dialysis was employed in the batch, shake cultures to determine the concentration of free metal ions and low molecular weight soluble complexes. Dialysis bags containing 2 ml (=1% culture volume) of deionized water were suspended in the shaking cultures for 5 h. This was experimentally determined as the optimum period for metal diffusion into a dialysis bag. Attempts to use *in situ* dialysis within the chemostat were unsuccessful due to the difficulties in equalizing pressure on both sides of the dialysis membrane.

Analytical Methods

Polymer was estimated by acid hydrolysis of dialyzed whole culture samples and assayed as glucose equivalents, as described previously [24]. Metals were determined by a flameless atomic absorption spectrophotometric method, as described previously (Rudd et al., in press). All glasswear used for metal samples was leached in 10% nitric acid for 24 h before use.

Mass Balance

From the results of the metal analysis, the following mass balance was constructed:

$$M_t = M_a + (M_{b_c} + M_{b_s}) + M_p + M_f \quad (1)$$

where M_t = total metal in culture
M_a = cellular uptake of metal
M_{b_c} = metal bound by capsular polymer
M_{b_s} = metal bound by soluble polymer
M_p = precipitated metal
M_f = free metal in solution.

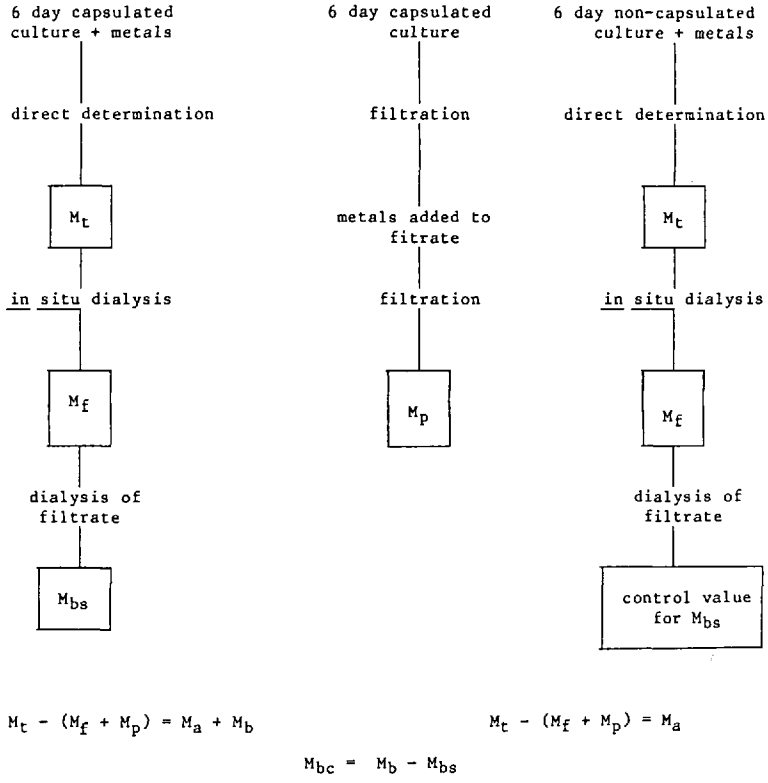


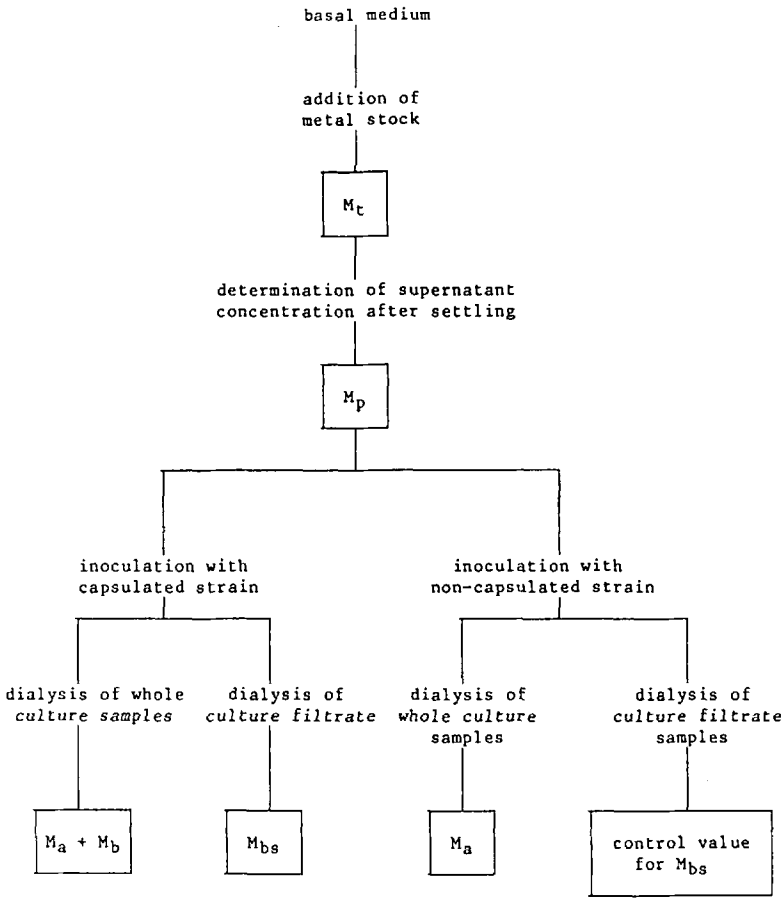
Fig. 1. Flow chart of sampling procedure for construction of a mass balance of metal uptake by a capsulated strain of *K. aerogenes* in batch culture at pH 4.5.

M_a was estimated as the metal retained on dialysis of the noncapsulated culture after subtraction of a blank value obtained by dialysis of pure metal solution under similar conditions. The total quantity of metal bound by extracellular polymer ($M_b = M_{b_c} + M_{b_s}$) was assessed by subtraction of the value M_a (corrected for cell numbers by division by the number of viable noncapsulated cells per ml of each sample) from the concentration of metal retained on dialysis of the capsulated culture. M_{b_s} was determined by subtracting the metal retained after dialysis of the noncapsulated culture filtrate from that of the capsulated culture filtrate, and M_{b_c} by $M_b - M_{b_s}$. Metal precipitation was assessed before inoculation of the culture medium, and where *in situ* dialysis proved impractical, M_f was obtained by subtraction of the sum of the fractions from M_t. Flow charts describing the sampling procedure are given in Figures 1 and 2.

Results

Mass Balance of Metal Uptake in Batch Culture at pH 4.5

The mass balance of metal uptake (eqn 1) in a batch culture at a low pH (4.5) is shown in Table 1. Cellular uptake, by adsorption onto cell walls or active uptake into the cytoplasm, of all metals was observed, copper and cadmium to the greatest extent and thallium the least. Capsular polymer bound a sub-



$$M_{bc} = M_b - M_{bs}$$

$$Mf = M_t - (M_p + M_a + M_b)$$

Fig. 2. Flow chart of sampling procedure for construction of a mass balance of metal uptake by a capsulated strain of *K. aerogenes* in batch and continuous culture at pH 6.8.

Table 1. Mass balance of metal uptake by a 5-day-old batch culture of *K. aerogenes* at a final pH of 4.5 in medium containing 10^{-5} *M* Cu, Cd, Co, Ni, Mn, and Tl.

mol l^{-1} metal ($\times 10^{-5}$)	Cu	Cd	Co	Ni	Mn	Tl
Cellular uptake	0.3	0.286	0.105	0.129	0.078	0.008
Capsular polymer binding	0.32	0.014	0	0	0	0
Soluble polymer binding	0.041	0	0	0	0	0
Precipitation	0.079	0.089	0	0.003	0.013	0.005
Free metal	0.385	0.92	1.06	1.09	1.05	0.467
Total metal by addition	1.133	1.309	1.165	1.222	1.141	0.48
Total metal by determination	1.25	1.34	1.19	1.03	1.15	0.738

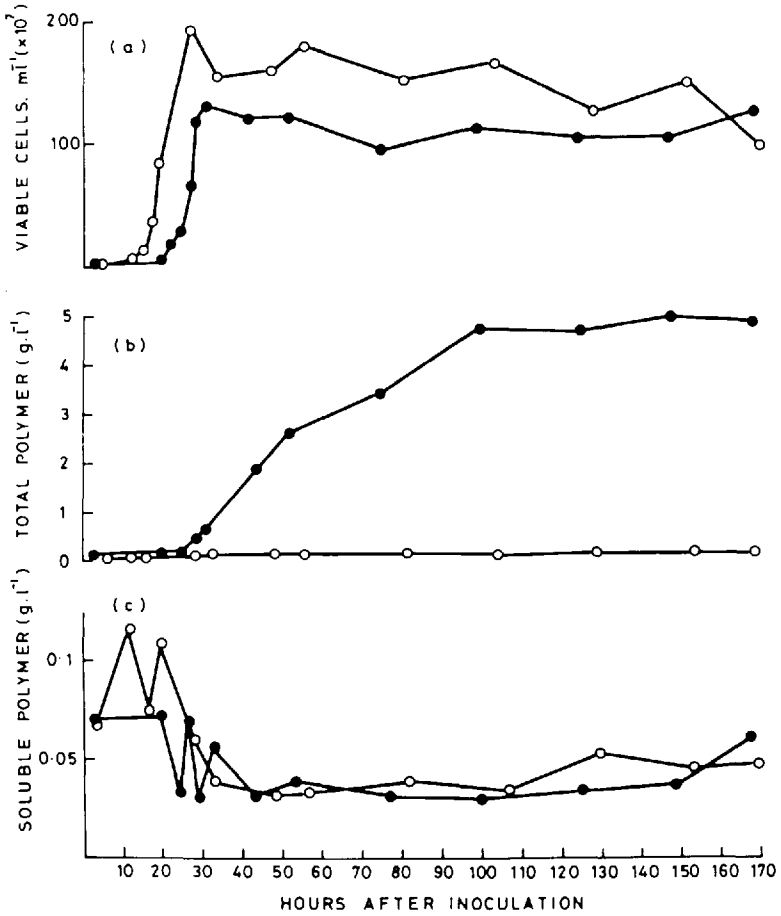


Fig. 3. Viable count (a), total polymer production (b), and soluble polymer production (c) of a capsulated strain (●) and a noncapsulated strain (○) of *K. aerogenes* inoculated into medium maintained at pH 6.8 containing 10^{-5} *M* Cu, Cd, Co, Ni, and Mn.

stantial amount of the copper present (26%) but only 1% of the total cadmium. Soluble polymer complexed 3% of the copper. Very little precipitation occurred at this pH. Copper and cadmium may have initially precipitated when the pH was higher and the precipitate had become incorporated in the capsular polymer matrix. Cobalt, nickel, manganese and thallium remained largely in an uncomplexed soluble form.

Recovery of 5 of the metals from the separated fractions compared well in total to that originally added, although the final concentration was higher than the nominal 10^{-5} *M*, probably due to evaporation of the cultures over the 5 days. Of the thallium added, however, only 48% was identified in the individual fractions, and 73% by direct determination. As the recovery of thallium was unreliable, probably due to adsorption onto the glassware, it was not included in further experiments.

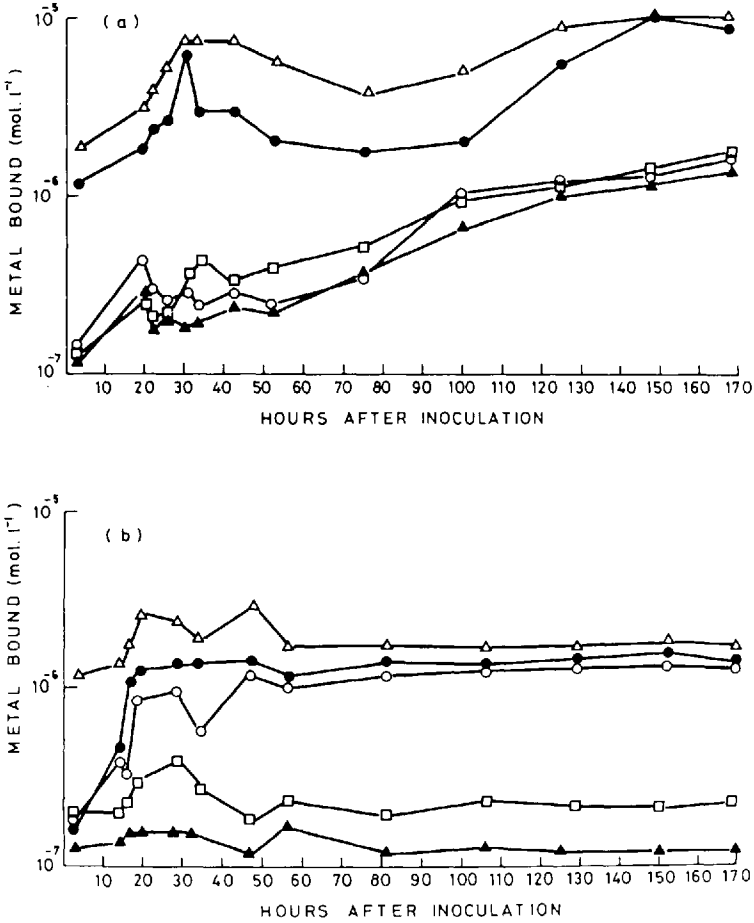


Fig. 4. Retention of Cu (△), Cd (●), Co (▲), Ni (○), and Mn (□) on dialysis of whole culture samples maintained at pH 6.8 of a capsulated strain ((a) = $M_b + M_a$) and a noncapsulated strain ((b) = M_a) of *K. aerogenes*.

Mass Balance of Metal Uptake in Batch Culture at pH 6.8

A mass balance could not easily be constructed for the batch culture grown in the chemostat, as the points of the 2 culture growth curves did not coincide (Fig. 3a). The lag phase was extended further in the capsulated culture than in the noncapsulated culture (by 8 h), hence data on both cultures are presented for visual comparison (Figs. 3 and 4). The values obtained for cellular polysaccharide and metal retention by the noncapsulated culture were used as a control for the capsulated strain.

A sample was taken 3 h after inoculation to assess initial uptake by passive cells (Fig. 4). Similar concentrations of copper, nickel, cobalt and manganese were retained by both cultures suggesting that uptake might have involved cells

rather than extracellular polymer. There was, however, a noticeable difference between the strains in the retention of cadmium; within this relatively short period, extracellular polymer complexed approximately 6 times as much cadmium as the cells alone. During the log phase, cellular uptake of copper and manganese, and more particularly cadmium and nickel by the noncapsulated strain (Fig. 4b), increased commensurately with viable count. The concentration of cadmium and nickel retained remained stable, but copper and manganese retention decreased at the end of log phase and stabilized at a concentration only slightly higher than that in the lag phase. Thus cadmium and nickel binding during rapid proliferation appeared to be of a more permanent nature than copper and manganese binding. Very little cellular uptake of cobalt occurred.

In each case except nickel, metal uptake by the capsulated strain (Fig. 4a) was higher than by the noncapsulated strain despite the fact that the cell numbers were lower (Fig. 3a). Retention of all the metals by the capsulated strain increased during stationary phase. Since the total cell numbers remained constant, the presence of extracellular polymer, which reached a maximum of 4,500 mg l^{-1} at 100 h growth (Fig. 3b), was probably responsible for this uptake. Production of soluble polymer by the capsulated strain was negligible (Fig. 3c). Metal concentrations determined in the dialyzed culture filtrates of both cultures showed no identifiable trends, and were generally of the magnitude of blank values for dialyzed metal solutions ($< 10^{-7}$ *M*).

Mass Balance of Metal Uptake in Continuous Culture at pH 6.8

Figure 5 shows the mass balance of metals at 5 different dilution rates in a chemostat. Cellular uptake of copper and cadmium was high, and increased with increasing dilution rate. At the highest dilution rate studied some accumulation occurred, as the concentration of metal retained by the cells exceeded the 10^{-5} *M* concentration in the influent medium. At the lowest dilution rate, 0.025 h^{-1}, copper and cadmium were bound by capsular and soluble polymers. This trend was observed also for manganese, where binding by capsular polymer exceeded that by the cells at the 0.05 h^{-1} and 0.025 h^{-1} dilution rates. These results indicated a shift in metabolism from intracellular uptake to extracellular binding as the dilution rate was decreased. Total extracellular polymer production ranged from 287.3–35,056 mg l^{-1} and soluble polymer from 0–28.1 mg l^{-1} at the 0.5 h^{-1} and 0.025 h^{-1} dilution rates respectively. There was evidence of slight cellular uptake and capsular polymer binding of cobalt, but the concentrations were low at all dilution rates. Nickel remained almost entirely in an uncomplexed soluble form except at the highest and lowest dilution rates where it was bound by capsular and soluble polymers respectively. Soluble polymer, although only present in very low concentrations, appeared to bind all 5 metals at the 0.025 h^{-1} dilution rate, especially nickel and copper. A comparison of the quantity of metal bound by the capsular and soluble polymers (Fig. 5) with regard to their concentrations (see above) suggests that the soluble form has a greater capacity per unit mass for metal binding.

Precipitation in the medium accounted for 51% of the total cadmium, 27%

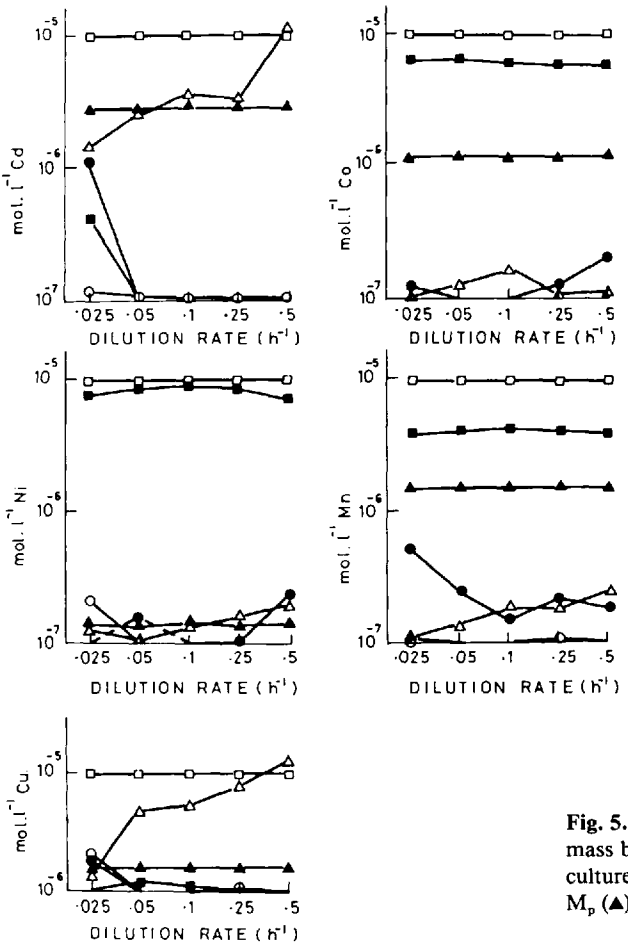


Fig. 5. Influence of dilution rate on mass balance of metals in continuous culture of *K. aerogenes*: M_t (□), M_f (■), M_p (▲), M_a (△), M_{b_c} (●), M_{b_a} (○).

of the manganese, 16% of the copper and cobalt, and 2.4% of the nickel. These figures were considerably higher at pH 6.8 than in the batch, shake culture at pH 4.5.

Discussion

Sublethal concentrations of heavy metals frequently retard the onset of bacterial growth. The difference observed in the extension of lag phase in the 2 cultures was unexpected, as it had been assumed that the presence of extracellular polymer might protect the capsulated cells against the toxic effects of the metal [3]. If, however, the ability to acclimate to toxic metals involves enzymatic reorganization within the exposed cell [1], the obstruction of ion influx by the capsule may prolong this process and hence the lag phase.

Uptake by resting cells showed little variation between the 2 strains with the

exception of cadmium. Adsorption of this ion in *Aerobacter aerogenes* cultures has been attributed to surface binding rather than active uptake [29]; the results here indicated that extracellular polymers may be involved in such surface binding. During log phase of the batch culture and at the higher dilution rates in continuous culture, however, considerable cellular uptake of cadmium was observed. Khazaeli and Mitra [17] have identified an inducible cadmium-binding protein in cadmium accommodated *Escherichia coli* cells which compartmentalized and thus detoxified the metal, allowing the cells to resume normal metabolic function. The uptake of cadmium by *K. aerogenes* may have occurred by a similar sequestering mechanism.

It has been suggested that nickel and cobalt antagonize magnesium uptake in *A. aerogenes* [29] and therefore compete for similar binding sites. Results of cellular uptake of nickel and cobalt in continuous culture, although low, indicate that there may have been mutual exclusion of these ions. The loss of copper and manganese from the noncapsulated culture in early stationary phase paralleled a slight decline in cell numbers, a phenomenon that has been observed also for copper and lead uptake by *Sphaerotilus natans* [26]. The effect of such self-inhibitory accumulation of even essential trace metals may be attenuated by the presence of capsular polymer.

The improvement in metal retention due to the presence of extracellular polymer has been demonstrated in batch culture. The gradual accumulation of all 5 metals suggested that the binding was nonspecific, as has been found for general binding of a wide range of cations by zoogloeal matrix [12]. Friedman and Dugan [13] found that the zoogloeal matrix-producing strain, *Zoogloea ramigera* 115, bound twice as much metal as the nonzoogloeal strain *Z. ramigera* 1-16-*M*. A similar ratio of metal retention was obtained in this study, with the notable exception of nickel. Uptake of this metal appeared to be inhibited by the presence of capsular polymer, which may have blocked binding sites on the cell surface. Brown and Lester [5] found that nickel bound more readily to activated sludge flocs that had been stripped of polymer than to untreated flocs. It has been suggested that nickel binds to soluble polymer in preference to the capsular form (Rudd et al., in press); binding of nickel by soluble polymer was again evident here.

The influence of pH on speciation and adsorption has been stressed by Nelson et al. [20]. Hydrogen ions will compete with other cations for binding sites, hence at low pH many potential metal binding sites are occupied. Metal uptake by *K. aerogenes* has been shown to be pH dependent; very little adsorption occurred at pH 4.5 compared with pH 6.8. Cheng et al. [8] found that the uptake of metals by activated sludge increased with increasing pH up to the level at which the metal hydroxides precipitated.

Metal removals from solution by the capsulated culture were of the order:

$$\text{Cu, Cd} > \text{Co} > \text{Mn} > \text{Ni}$$

which showed a similarity to the metal affinity of *Z. ramigera* 115 flocs [12]:

$$\text{Fe} > \text{Cu} > \text{Co} > \text{Ni}$$

and to affinity series constructed for laboratory scale activated sludge biomass [8, 27]

$$Cr > Cd > Ag > Pb > Zn > Cu > Ni, Co, Mn, Mo$$

$$Pb > Cu > Cd > Ni.$$

Cobalt, manganese and nickel generally have poor removals in the activated sludge process [27], which may be associated with their high solubility, evident from the level of precipitation determined in continuous culture. Manganese and nickel have been found to associate with nonsettleable solids rather than mixed liquor biomass [27]. Preferential binding of this nature results in metals adsorbed to small particles or complexed with ligands such as soluble polymer being discharged in sewage effluent rather than removed during final settling.

The results presented suggest that process parameters such as the physiological age of the culture and dilution rate may affect metal removal. In batch culture, overall metal removal was most effective during late stationary phase, when maximum polymer production appeared to enhance the cellular uptake. In continuous culture, maximum removals generally occurred at the highest and lowest dilution rates where intracellular and extracellular uptake predominated respectively. Metal removal may occur to a greater extent in cultures with long cell retention times as the biomass may be more tolerant of toxic concentrations of metals if they are immobilized extracellularly by physiochemical mechanisms rather than internally by biological pathways. Moreover, the presence of extracellular polymer may indirectly increase metal removal by improving the flocculation and settling properties of the biomass [4], thus reducing loss of cations associated with the effluent suspended solids.

In practice, greater metal uptake has been reported at a 5-day sludge age than at a 1-day sludge age by Nelson et al. [20], who attributed it to increased polymer production. Casey and Wu [7] found that increased concentrations of capsular material improved the metal adsorption capacity of activated sludge bacteria. Other work has demonstrated that prolonging the sludge age [25] or dilution rate [24] has the effect of reducing the concentration of soluble polymers which tend to exacerbate metal removal rather than ameliorate it.

This investigation has shown that capsulated cells of *K. aerogenes* are capable of retaining greater concentrations of metals than an equivalent number of noncapsulated cells, and although only a fraction of some of the metals added was retained, in comparison to typical environmental concentrations such metal removals may be of considerable significance in the activated sludge process of sewage treatment.

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