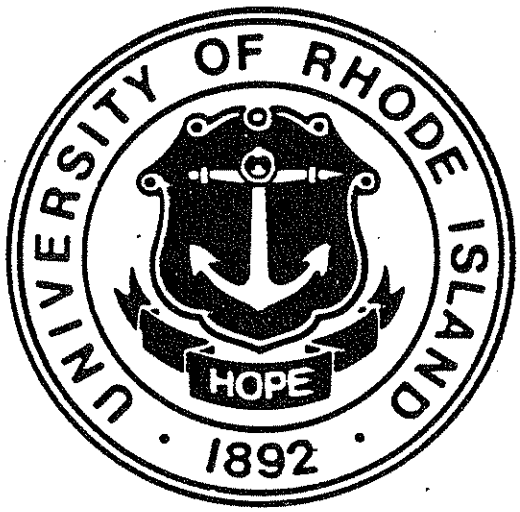
RHODE ISLAND
WATER RESOURCES CENTER



COMPLETION REPORT FY-1987 PROGRAM

A MICROBIAL METHOD FOR REMOVAL OF METALS, CUTTING OILS AND CYANIDE FROM POLLUTED WATER—PHASE I

by

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COMPLETION REPORT

Title: A Microbial Method for Removal of Metals, Cutting Oils and Cyanide From Polluted Water - Phase I.

Duration: June 1987 to May 1988

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INTRODUCTION

Regulations limiting the discharge of toxic metals and cyanide into public sewers have been in effect for three years but the majority of affected platers and finishers are not in compliance with these regulations. Many of the larger firms meet discharge standards or are expected to do so in the near future.

The magnitude of the problem is seen by the amount of heavy metal entering the Narragansett Bay Commission sewer system in recent years. The peak was in 1981 with 956,099 pounds release with a drop in 1985 to 409,657 pounds. The interim goal is to reduce this volume to about 200,000 pounds within a year. The existing technology for solution of this problem by chemical means is expensive. One of the larger companies was forced to spend $500,000 for a pretreatment system. This type of a solution to the problem is beyond the capability of the smaller operator who does not have the capital resources for even a 5th of this type of investment. The ultimate result is that without a less expensive system these small companies will be forced out of business. This project is aimed at the small operation to provide to these companies a relatively simple, inexpensive but effective means of pollution control. The project while of extreme potential value to Rhode Island can also be applied to the same problem in other states.

A. The FY 1987 Objectives Phase I Metal Removal:

1. Expand resistance spectrum in one isolate, if possible, to include resistance to Cu, Pb, Ag, Cr, Zn, Cd, Ni and Hg.

2. Determine the kinetics of metal removal.

3. Determine the saturation level of cell mass.

4. Determine the effect of nutrients on metal removal.

5. Perform bioreactor studies with simulated and real waste for metal removal.

6. Determine the presence or absence of plasmid DNA which is linked to the objectives of Phase II.

B. Phase I Results and Discussion

Objective 1.

Using a serial enrichment-adaptation procedure we have been able to expand the resistance spectrum of Arthrobacter HC823 to a combined mixture of eight metals, each at a concentration of 50 ppm. The eight metals are: Cu, Pb, Ag, Cr, Zn, Cd, Ni, and Hg. This is a combined total metal ion load of 400 ppm of metal ion in the test system. We are delighted to be able to achieve this resistance pattern in one isolate.

In addition, we have determined the metal resistance of a natural isolate identified as a Flavobacterium sp. and a member of the genus Pseudomonas labeled strain PxR824, isolated from heavily metal polluted sediment. Tests have not been run to enhance the resistance of these isolates. Those metal listed as R (resistant) were resistant to 50 ppm of the metal while those listed as S (sensitive) would not grow in the presence of 1 ppm of the metal (Table 1.).

On isolation, the Flavobacterium sp. required 1 ppm of zinc for growth in solid or liquid medium. After sub-culture the organism no longer shows a Zn requirement for growth.

Table 1. Resistance spectrum of two environmental isolates.

Cation	Flavobacterium sp	Pseudomonas HC824
Pb	R	R
Zn	R	R
Cd	R	R
Cr	R	S
Cu	R	R
Ni	R	R
Ag	R	S
Hg	S	S

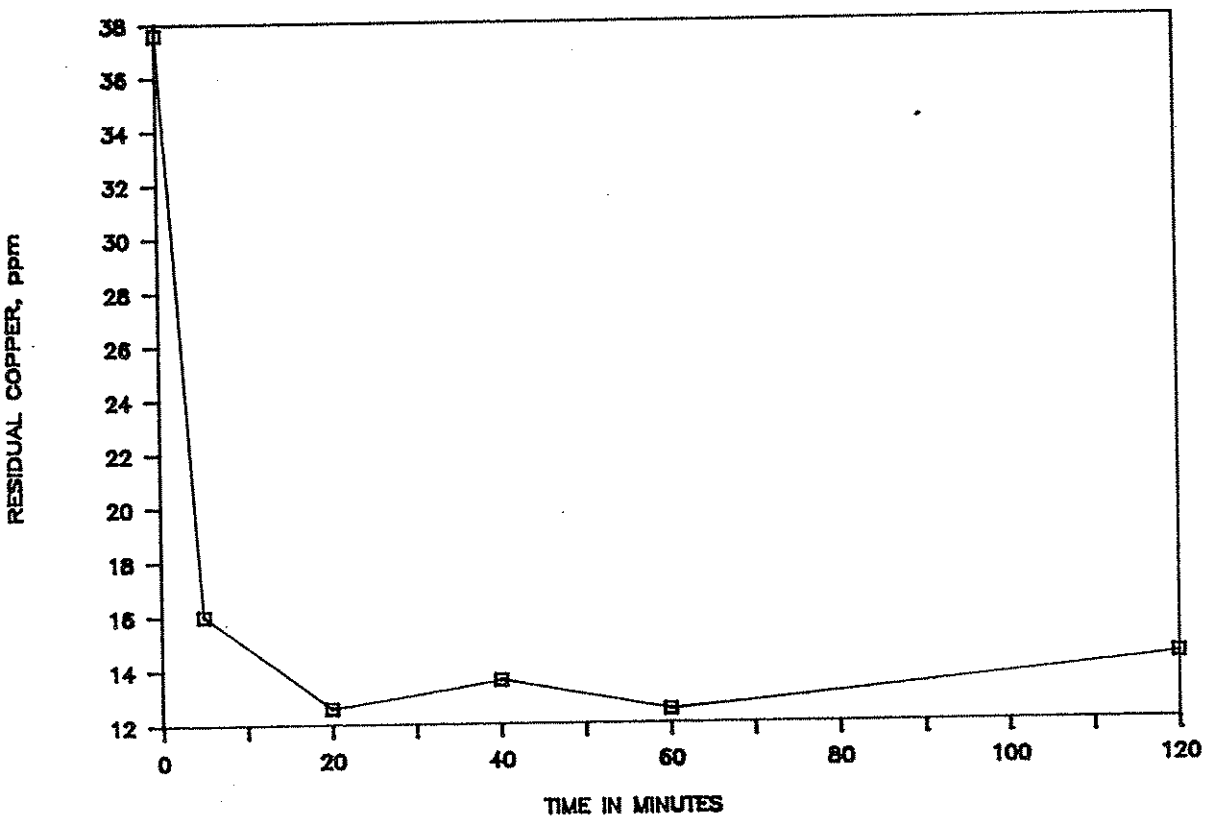
Objective 2.

Kinetic studies were initiated in November using Cu as the first metal in non-nutrient supplemented experiments. The first experiments indicate a much more rapid removal of the metal from a microbial suspension in distilled water than anticipated. We find the same rapid uptake by Arthrobacter HC824 with Pb, Zn, Ni, and Cr (Figures 1-5). Uptake of Ni and Cr are initially rapid for the first 5 minutes then the uptake rate is reduced. These differences in kinetics suggest different mechanisms associated with cell binding. Also, it is seen from these data that binding or uptake is highly efficient with Pb and Zn, less so with Cu and Ni, and poor with Cr.

Arthrobacter HC824 was tested in a mixture of Pb, Cu, Zn, and Ni suspended in distilled water plus yeast extract (Figure 6), and the metal content of the aqueous phase determined as an index of metal removal from solution. Lead was rapidly cleared from the aqueous phase. The efficiency of metal removal was less for Cu, Zn and Ni.

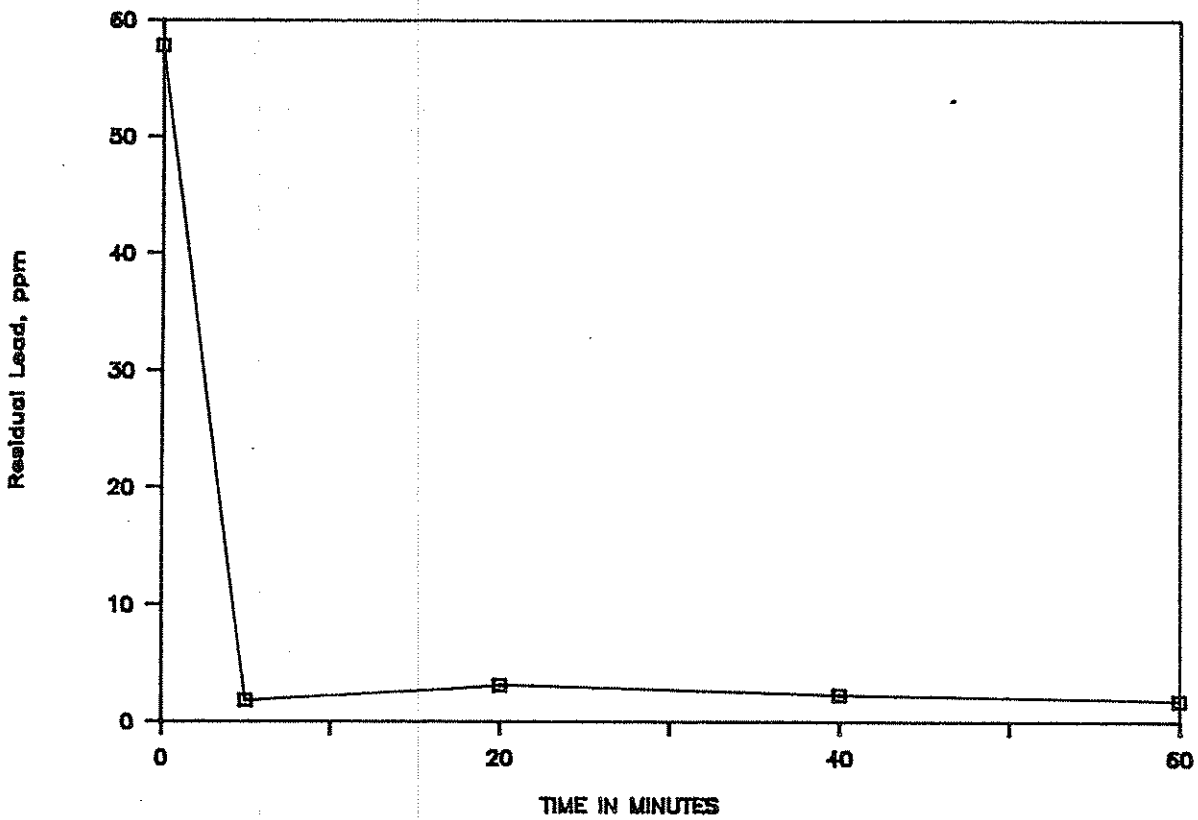
The first experiment with Pseudomonas PxR was conducted in a dilute mineral medium with glucose as the carbon source, and amended with Cu at 50 ppm. The cell mass (mg/ml dry weigh basis) was varied from 12-48 mg/ml (Figure 7). In this 3 hour

Figure 1. Kinetics of Cu uptake By Arthrobacter HC824



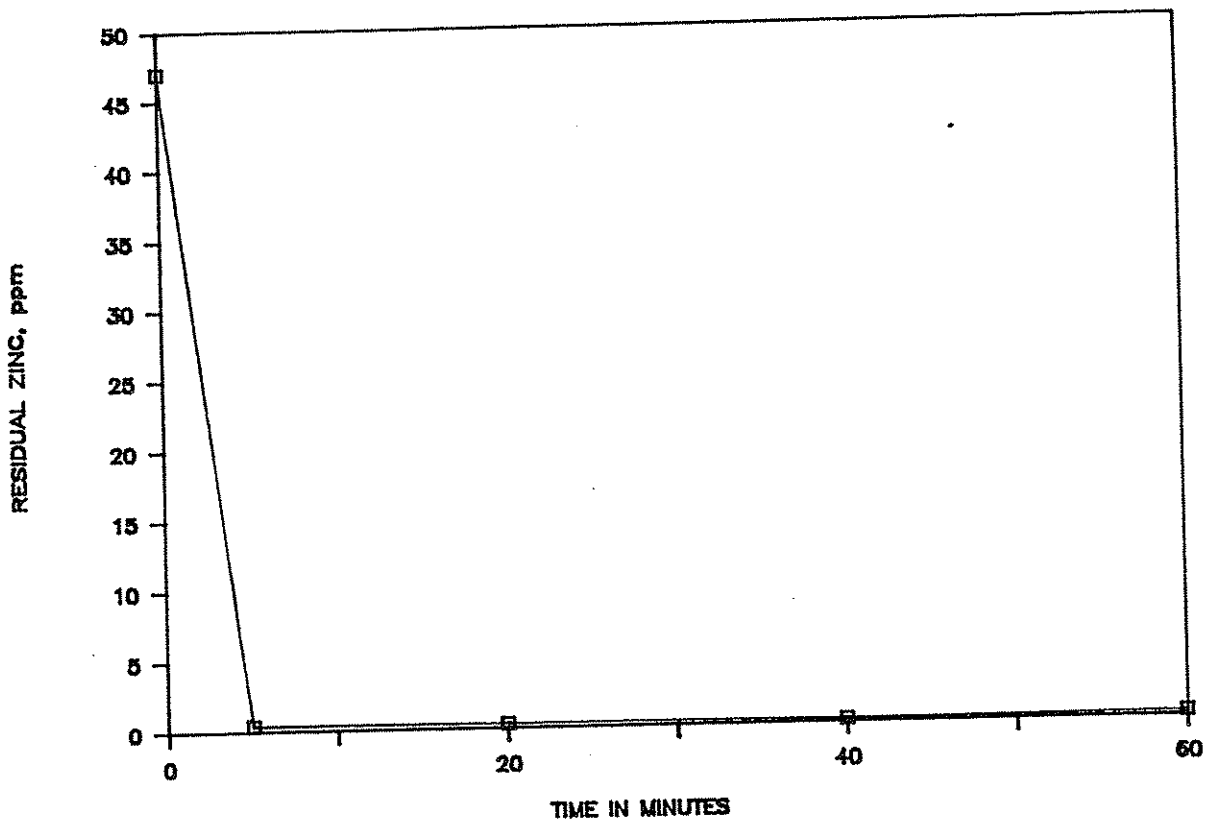
9.2 mg/ml of cells in distilled water + 0.01% yeast extract

Figure 2. Kinetics of Pb uptake by Arthrobacter HC824



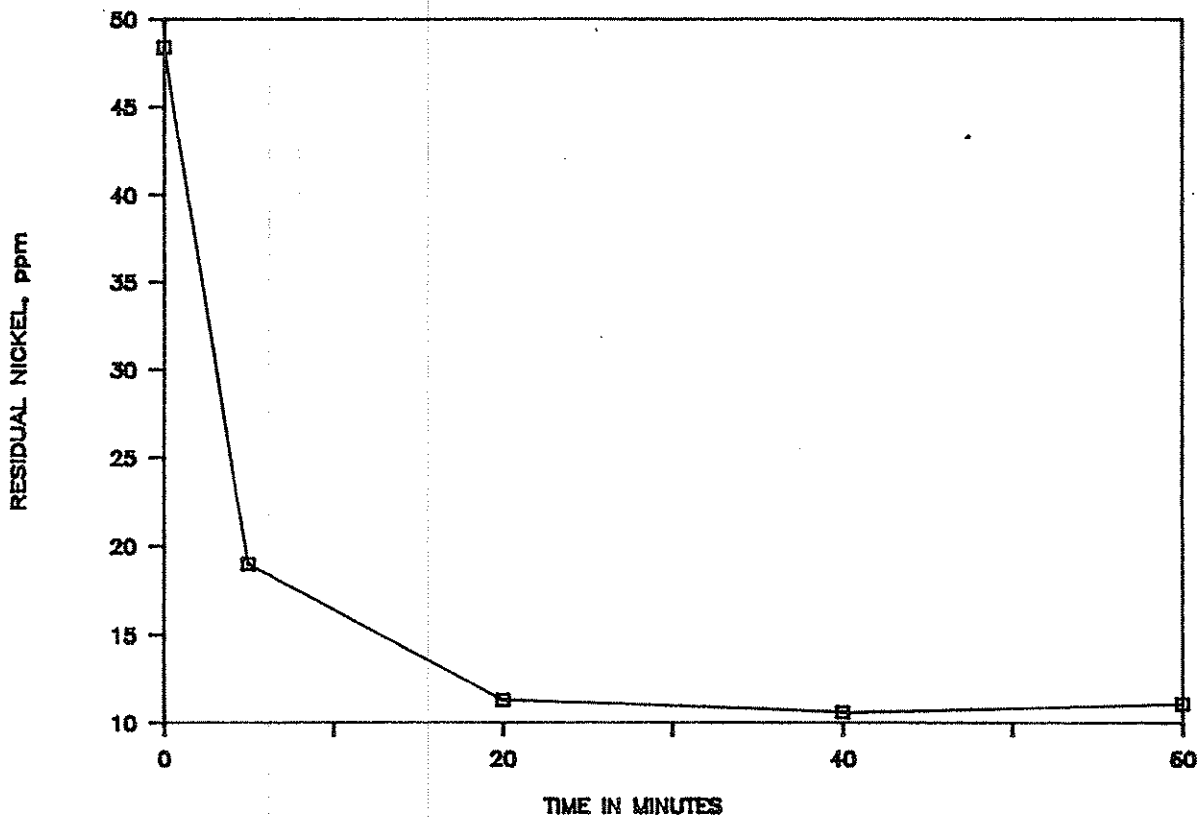
7.5 mg/ml of cells in distilled water + 0.01% yeast extract

Figure 3. Kinetics of Zn uptake by Arthrobacter HC824



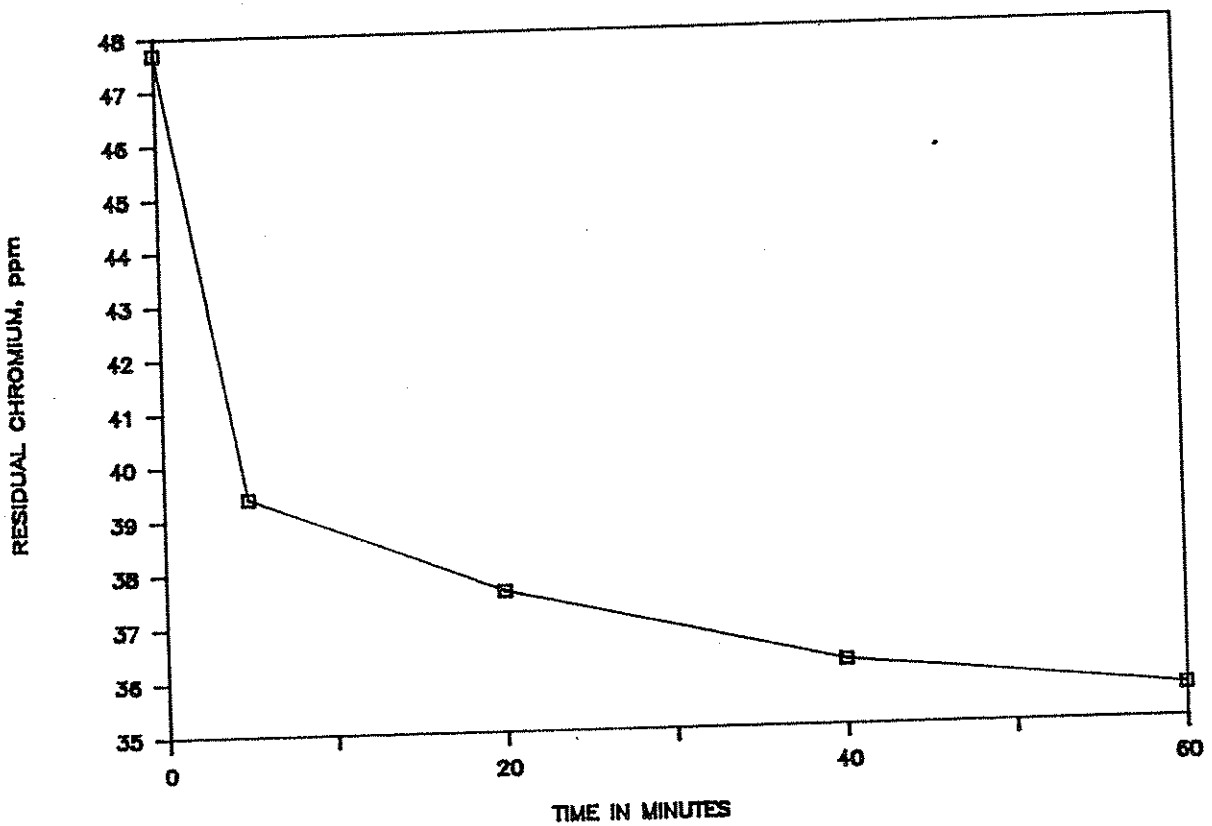
6 mg/ml of cells in distilled water +0.01% yeast extract

Figure 4. Kinetics of Ni uptake by Arthrobacter HC824



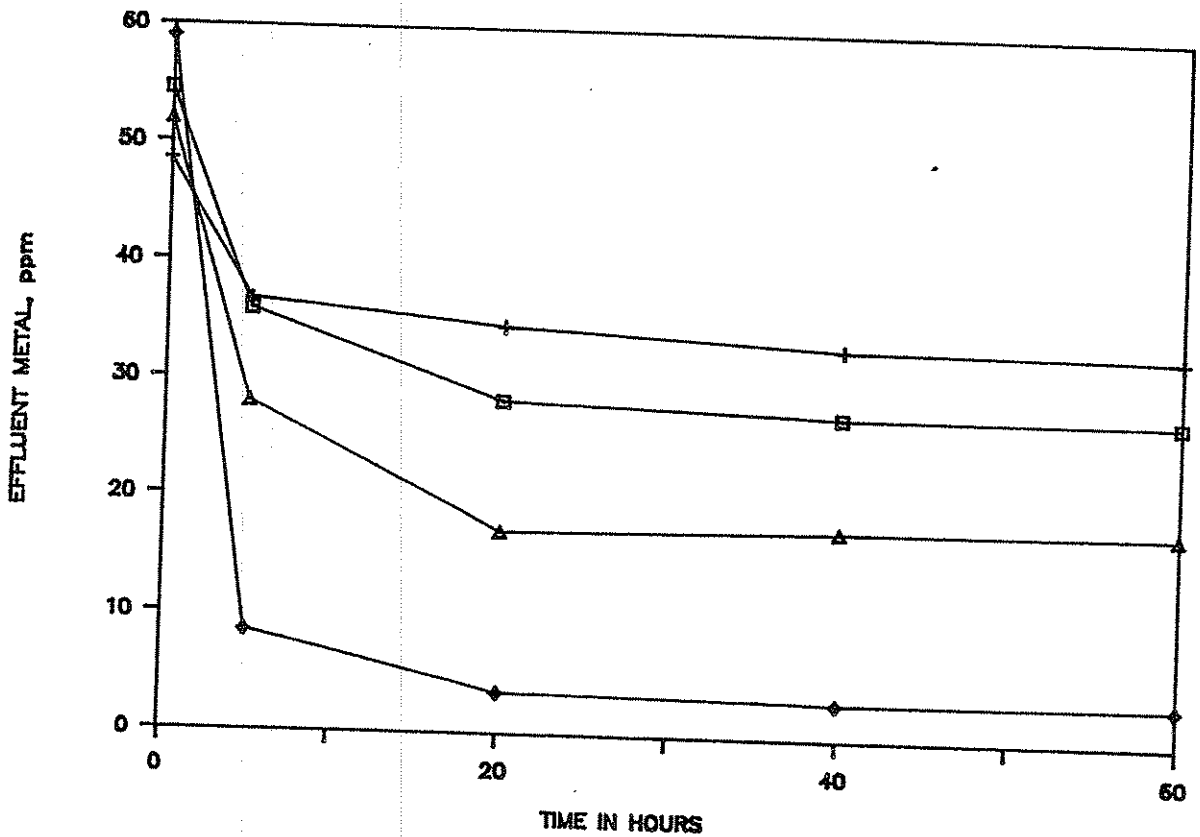
6.5 mg/ml of cells in distilled water + 0.01% yeast extract

Figure 5. Kinetics of Cr upatke by Arthrobacter HC824



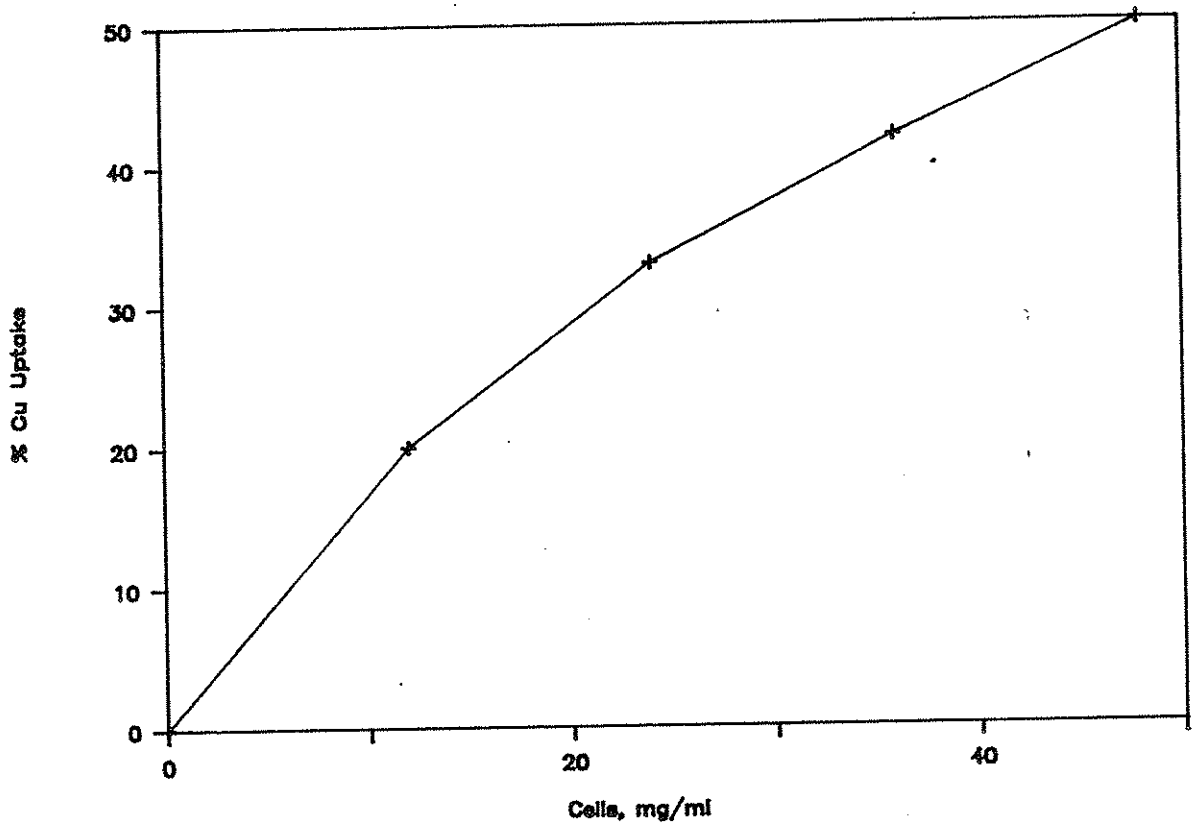
6.5 mg/ml of cells in distilled water + yeast extract

Figure 6. Kinetics of uptake of a metal mixture by Arthrobacter HC824 in distilled water plus yeast extract



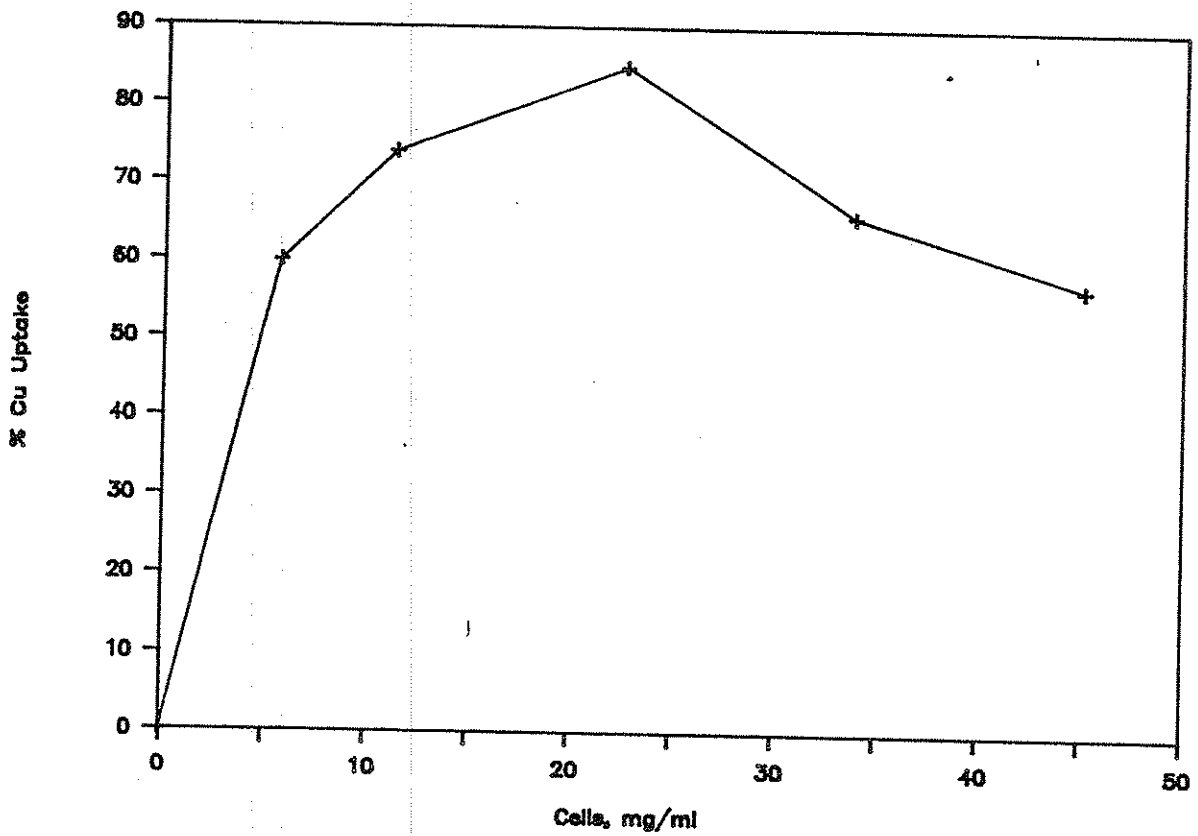
15.2 mg/ml cells; ◆Pb, ▲Cu, □Zn, +Ni

Figure 7. Percentage Cu uptake by Pseudomonas PxR in dilute mineral medium with glucose as the energy source



14.8 mg/ml of cells

Figure 8. Percentage of Cu uptake by Pseudomonas PxR in distilled water plus yeast extract



14.8 mg/ml of cells

experiment maximum uptake (50 %) was obtained with the highest cell mass, and there was a different relationship between cell mass and percentage metal uptake. A similar experiment experiment performed in distilled water (Figure 8), does not show an increased metal uptake at cell masses above 22.6 mg/ml, which was optimal for Cu removal (85%). These experiments confirm earlier observations that metal removal from aqueous solution are inhibited to some extent in more complex media and media with even modest quantities of other inorganic salts.

Solanellas and Bordons (1988) used Pipes Buffer (piperazine-N,N'-bis (2-ethanesulphonic acid) in their copper uptake studies because of it negligible metal-chelate properties. We compared Cu uptake by Pseudomonas Pxr in Pipes and our distilled water-yeast extract system with 21 mg/ml of cell suspension. In the 3 hour experiment 83% of the Cu was cell bound in water versus 90% in Pipes. A test with Flavobacterium sp. at 14.8 mg/ml in Pipes, removed 71% of the added Cd.

Objective 3.

Early studies indicated that saturation of the cell mass with metal occurred at a metal:cell dry weight in the range of 1:1.4 to 1:2.8 mg metal:mg cell dry weight. From this data we had a guide to the amount of cell mass needed to clear a waste water. It is now apparent that the value for cell saturation varied depending upon the organism used in the experiments.

Objective 4.

We know that glucose used as a energy source (1%) enhances metal removal from a water solution but is not as effective as small quantities of yeast extract (0.01%). Using a mixture of glucose and yeast extract offers no advantage over yeast extract alone. A recent study by Brynhildsen, et. al. (1988) demonstrated that glucose concentration had a significant effect on the sensitivity-resistance of a Klebsiella sp. to Cd, Cu, Hg, and Zn.

Table 2. Effect of Nutrient Supplements on Clearing of Cu, Cr, Ag and Pb From Water

Metal	Glucose 1%		Yeast Ex .01%		Yeast Ex .1%		PO4 0.1M	
	1	2	1	2	1	6	1	2
Cu	4.09	.41	4.43	.26	4.58	.27	.18	4.36
Cr	4.67	.17	4.67	.21	4.58	.27	4.75	.03
Ag	2.4	.07	2.7	.1	3.3	.05	.4*	.1
Pb	4.3	1.5	2.0	3.2	1.3*	3.4	.4"	3.2

M:C	1:2.6	1:1.5	1:1.4	1:0.7

1= Water phase
2= Cells
* ppt before cells added
" ppt at end 4 hrs incubation

Phosphate would appear to enhance Cu and Pb cell association but lowers the association of Cr and cells. Phosphate also has a chemical reaction with Ag (apparently the formation of silver orthophosphate which is insoluble) which removes the silver from solution.

Yeast extract is a complex mixture of compounds which are to a large extent amino acids, peptides and vitamins. It is a very promising supplement for this project in that low concentration is required for enhanced activity and it is a relatively cheap product.

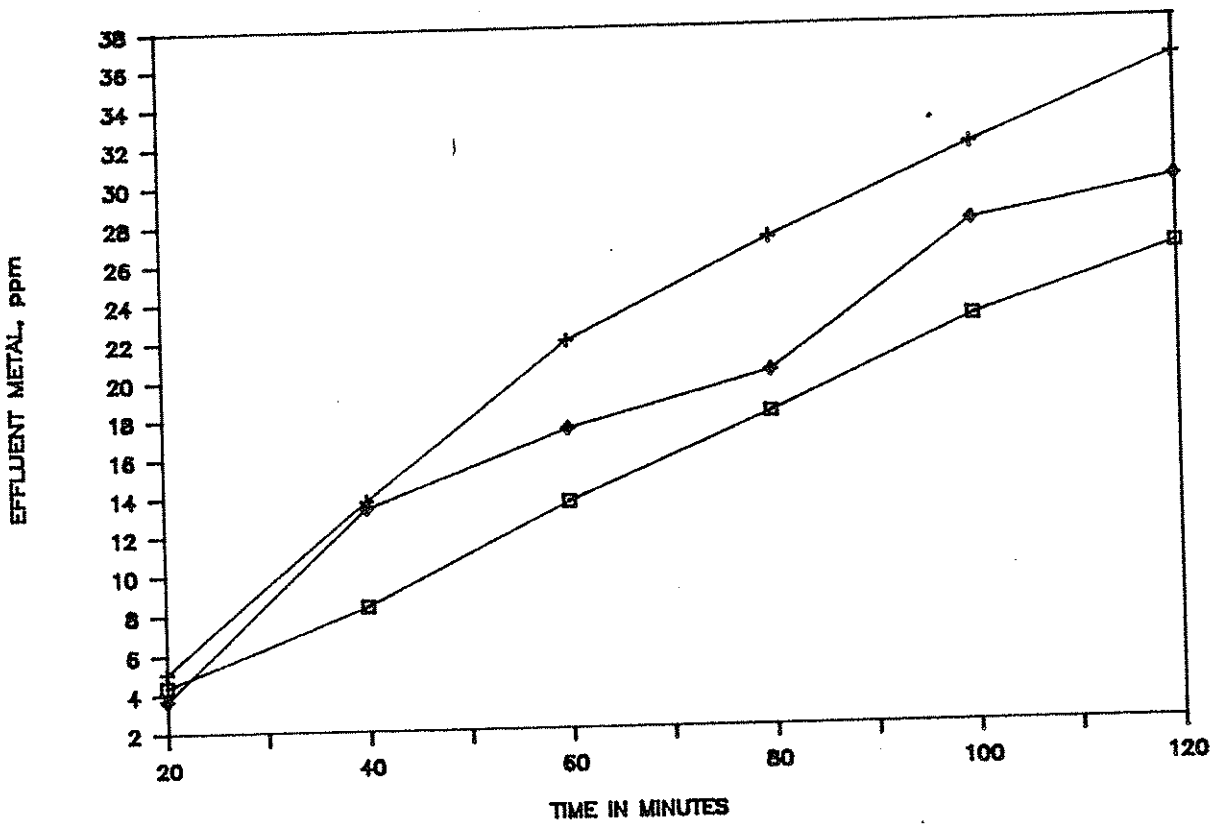
Objective 5.

Due to serious problems associated with the relocation of our research laboratory this phase of the project has been delayed since mid-January. One bioreactor test was run in early January with Arthrobacter HC824 at 10 mg/ml final cell dry weigh. The cells suspended in 300 ml of distilled water yeast extract were mixed with 400 ml of water containing 50 ppm each of Cu, Ni and Zn. This mixture was mixed for 20 minutes, then a feed of the same metal solution was fed to the bioreactor at 6 ml/minute. At the time the feed was started, the bioreactor was pumped at high speed across a membrane in tangential flow filter. The cell slurry retentate was returned to the bioreactor and the permeate (aqueous phase) went to discharge at a rate of 6 ml/minute. Thus, the bioreactor maintained a constant volume and constant cell mass. The results (Figure 9) showed a steady build-up in the metal content of the permeate over the 100 minutes of system operation. However, metal was still being removed from the aqueous phase as the permeate level never reached input metal concentrations of 50 ppm.

The experiment was repeated using four metals, Cu, Cr, Ni, Zn, and Ag, with a lower feed rate of the waste metal solution (3 ml/minute) and a cell mass of 8 mg/ml. The results in (Figure 10) demonstrated a good removal of Ag from the aqueous phase and poor but fairly constant removal of the other metals.

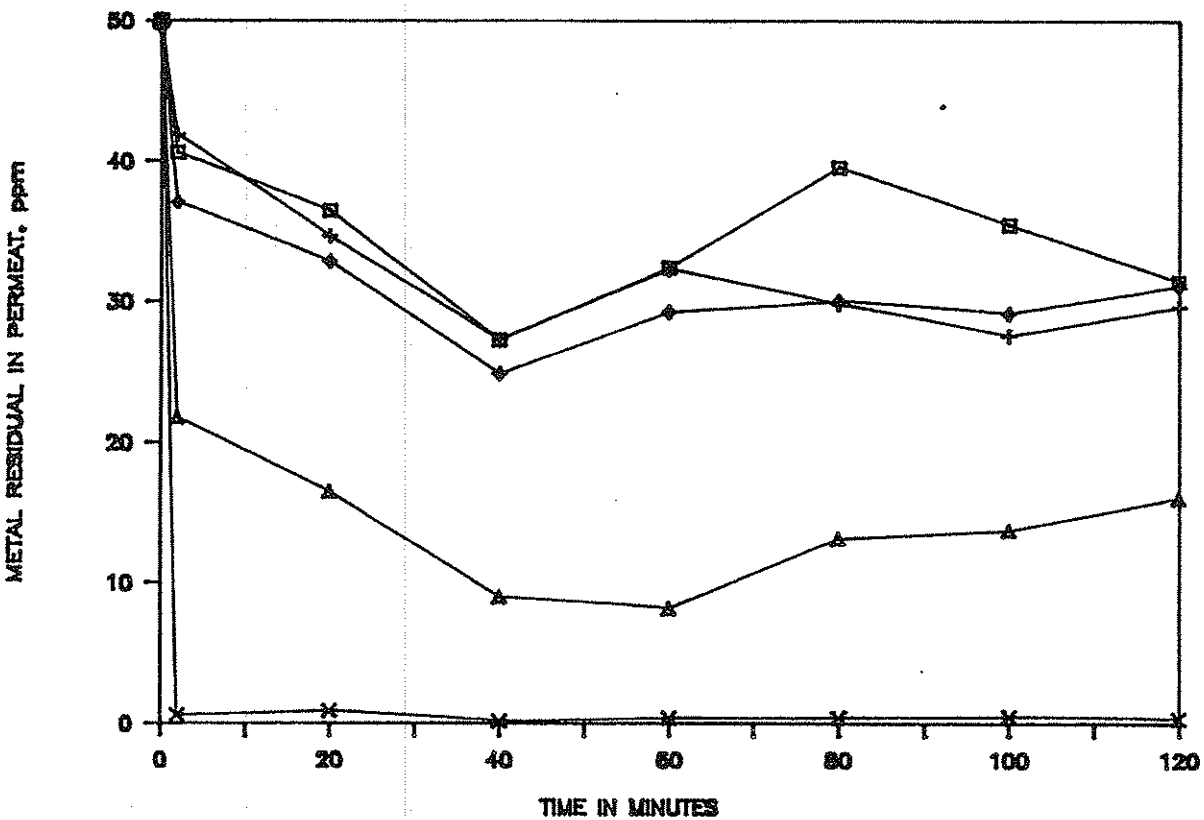
The data from other studies suggests that a much greater cell mass must be used in the bioreactors. Our objective in Phase II of this project will be the use of cell dry weights in the range of 20-30 mg/ml.

Figure 9. Bioreactor fed mixed metals Cu, Ni, and Zn



10 mg/ml Cell mass, feed rate 6 ml/min, feed atart 20 min.
□ Cu, ◇ Zn, + Ni

Figure 10. Bioreactor fed mixed metals, Cu, Cr, Ni, Zn, and Ag



8 mg/ml cells, 600 ml volume, fed at 3 ml/minute

Objective 6.

A variety of different digestion methods have been tried for the isolation of plasmid DNA from Arthrobacter HC824. All of these attempts have isolated only chromosomal DNA. In a recent test using a modification of the alkaline digestion method, there is evidence for a very high molecular weight plasmid near the chromosomal DNA region of the gel. Further tests during Phase II of this project will be done to confirm this observation.

At least one plasmid has been detected in the Pseudomonas PxR strain by the alkaline lysis method. The molecular weight of the plasmid is approximately 11 kb. It was cut by EcoRI into two fragments, 9kb and 2 kb, which demonstrated that the plasmid has a single EcoRI restriction site. In addition, the PxR gels show a high molecular weight band, which may indicate the presence of a large molecular weight plasmid. The alkaline lysis method has been used for a plasmid screen of clinical isolates of Pseudomonas aeruginosa and of Ps. fluorescens. These screens have not detected plasmids in these strains. Work will continue during Phase II of this project to isolate and restrict this potential plasmid from Ps. PxR.

In order to confirm the function of the plasmid(s) in the Ps. PxR strain, it is necessary to transform it into a suitable recipient strain and test for the retained function and plasmid presence in the recipient strain. Since antibiotic resistance is normally coded on the same plasmid as metal resistance, we have two markers to use in the transformation studies. We have screened metal and antibiotic resistance in three potential recipient organisms, Ps. aeruginosa, Ps. fluorescens and E. coli HB101. The results of the metal and antibiotic resistance screens are shown in Tables 3 and4. The two Pseudomonads are not good candidates as recipients as the patterns are similar to Ps. PxR. E. coli HB101 may be a good transformation recipient due to the Cd, Cr, Hg, ampicillin and chloramphenicol sensitive markers not present in the PxR strain.

Table 3. Metal resistance of strains

Agents and Concentration	E. coli HB101	Ps. PxR	Ps. fluorescens	Ps. aeruginosa
Control	R	R	R	R
Pb, 0.5 mM	R	R	R	R
Zn, 2 mM	R	R	R	R
Cd, 0.5 mM	S	R	S	R
Cr, 2 mM	S	S-R	S	S
Ni, 2 mM	R	R	R	R
Cu, 2 mM	R	R	R	R
Hg, 0.5 mM	S	R	S	S
Ag, 0.5 mM	S	S	S	S

Table 4. Antibiotic sensitivity pattern of strains

Agent	E. coli HB 101	Ps. PxR	Ps. fluorescens	Ps. aeruginosa
Control LB+Amp 50 ug/ml	S	R	R	R
LB+Kan 50 ug/ml	S	S	S	S
LB+Tet 10 ug/ml	S	S	S	R
LB+Chl 30 ug/ml	S	R	R	R

Additional Observations

While not included as an initial objective of this project, it has become apparent that the metal binding organisms may produce and secrete into the metal compounds which function as metal chelate agents. Ps. HC824 was grown in Trypticase Soy Broth in the presence of 5 ppm of Cd, the cells removed and the supernatant collected for analyses. The protein content of the supernatant was estimated by the Warburg-Christen method by measuring the Absorbance at 260 and 280 nM and using their table to estimate protein. The supernatant was divided into aliquots to which were added varying (0-50 ppm) of the metals under study in this project. A UV-Vis scan of the native supernatant with and without added metals was performed. Comparison of the scans indicates the possible chelation of Cd, Cu, Pb, Cr, and Zn by extracellular products of the organism, but not the chelation of Ag, Ni or Hg. The spectral shifts in the presence of metal are metal specific and represent the formation of new peaks, magnification of peak shoulders, and changes in Absorbance maxima.
It is too early to equate these findings with metal removal from aqueous solution but it suggests that events other than metal uptake are associated with the clearing of metals from aqueous solutions,

C. New Literature Summary and References

Erardi, et. al. (1987) used a copper tolerant strain of Mycobacterium scrofulaceum to remove copper from culture medium by sulfate-dependent precipitation as copper sulfide. A derivative of this strain which lacked a 173-kilobase plasmid did not precipitate copper. The plasmid-carrying strain had a sulfate-independent copper resistance mechanism.

Chlorella stigmatophora grown in artificial seawater produced cell wall polysaccharides which dissolved in the growth

medium (Kaplan, et. al., 1987). This polysaccharide had a varying complexing capacity for Zn, Cd, Pb, and Cu.

Brynhildsen, et. al. (1988) found that the nutritional state of an organism has a profound effect on its sensitivity to metals. Metals taken up by an energy-driven transport system may be less toxic under conditions of carbon starvation. The toxicity of Cu, Cd, and Zn to a Klebsiella sp was affected considerably by the carbon concentration, whereas the toxicity of Hg is independent of carbon concentration.

Solanellas and Bordons (1988) surveyed copper accumulation by resting cells of copper-resistant bacteria isolated from sewage sludge. Their best strain for accumulation was a Bacillus which retained copper at up to 3.8% of its cell dry weight, in the absence of glucose.

Belliveau, et. al. (1987) reviewed metal resistance and accumulation in bacteria, and in this paper summarize most of the current data and concepts of metal transport, resistance and accumulation.

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D. Conclusions

The studies with PS. HC824 demonstrate that it is possible to enhance the metal resistance pattern of an environmental isolate with limited resistance to a series of heavy metals, to an organism with high resistance to the metals. It is found that environmental isolates obtained by enrichment isolation from heavily polluted sediments readily yield bacteria which carry multiple metal resistance to the metal pollutants of the area.

We are surprised at the rapid metal uptake by metal resistant bacteria. The kinetics of uptake vary with different isolates and with different metals, but the bulk of the metal which becomes cell associated occurs within a few minutes after addition of the organism. Of the metals studied Pb, Cu, Zn and Ni are the easily removed, whereas Cr is the most difficult.

Initially it was thought that the metal-cell saturation levels were constant. However, it is now apparent that this is a somewhat more variable and complex relationship. It is apparent that actively growing cells will accumulate or bind metal, but that resting cells with a minimum of nutrient supplement give superior results in metal removal. The non-chelating buffer, Pipes, seems to be a an ideal system with Pseudomonads for the removal of metal.

The bioreactor visualized for this system shows promise but will require additional study, particularly using reactor cell mass in the range of 20 mg/ml or greater. The results with clearing of Ag from solution are very good even in the preliminary experiments.

It is becoming apparent that the strains we are using for this study probably carry plasmids which carry the genetic information for specific metal resistances. This may allow us to optimize the system by carrying genetic information from more than one strain into a common organism to clear the various metals from solution