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# Cadmium stress studies: Media development, enrichment, consortia analysis, and environmental relevance

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**Abstract**

The effects of Cadmium (Cd) toxicity on bacterial consortia originating from an-aerobic sewage sludge and cultivated under differing enrichment conditions were studied. Cultures were enriched in minimal media developed specifically for Cd stress studies. At inoculation all Cd was soluble in free ion or chelated form. Electron donors and acceptors were varied to obtain each physiological enrichment type. Adaptation leading to higher levels of Cd resistance of the consortia over time was observed under all physiological conditions. Initial and increased Cd tolerances were consistently greatest in multiphysiological enrichments (MPH). Sulfate reducing (SRB), methanogenic (MET), and fermentative (FRM) enrichments had less tolerance however, the level of tolerance to the Cd varied from one inoculation to the next. The Cd remained soluble as free Cd in MPH and FRM conditions and was precipitated significantly in SRB and moderately in MET conditions. Denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified 16S rRNA of the SRB, MPH, and FRM enrichments were followed over time. The consortia underwent succession under all physiological conditions when compared with the profile of the inoculum. Microbial population diversity decreased as the consortia were subcultured. The effects of chelators in the MPH medium were also evaluated. The addition of chelators transiently decreased toxicity. Effects of MPH medium on the Cd sorption capacity of soil were evaluated. Microbial growth decreased the amount of Cd left in solution.

*Abbreviations:* ATCC – American Type Culture Collection; DGGE – denaturing gradient gel electrophoresis; DNA – deoxyribonucleic acid; EDTA – ethylenediaminetetraacetic acid; ESI-MS – electrospray ionization mass spectrometer; F-A – fermentor physiological type analysis; FRM – fermentative enrichments; HCl – hydrochloric acid; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer); ICP-AES – inductively coupled plasma atomic emission spectroscopy; SRB – sulfate reducing enrichments; SRB-A – sulfate reducing bacteria physiological type analysis; M-A – methanogenic physiological type analysis; MET – methanogenic enrichments; MPH – multiphysiological enrichments; PCR – polymerase chain reaction; rRNA – ribosomal ribonucleic acid; SC – subculture; SDS – sodium dodecyl sulfate.

## Introduction

Cd has been reported to be more mobile than most toxic metals due to its low affinity for soil (McBride 1994). However, the chemistry of Cd and its transport in the environment is complex. Most Cd applied through phosphoric fertilizers to sandy soils will stay mobile due to a lack of adsorption sites on soil surfaces, and hence the risk of leaching to groundwater or uptake by plants is high (Mann & Ritchie 1995). However, organic matter, hydrous oxides of iron, aluminum, manganese, and clay minerals in soil may retain Cd in non-mobile or unavailable forms. The use of microbial processes in environmental restoration

requires an understanding of how the growth amendments added to the site will effect the interactions of the Cd with the soil structure. Past studies have shown very different results due to differing soils and medium components. In one study the Cd was mobilized due to a pH drop to 5.5 (Chanmagathas & Bollag 1987) while in another inoculation did not change the solubility of Cd (Stephen *et al.* 1999).

Microbial populations generally resist Cd toxicity by three processes (Ford *et al.* 1995). The first involves the secretion of a polymer, protein, or other component that sequesters Cd in the extracellular media (Khazaeli & Mitra 1981; Holmes *et al.* 1997; Pazirandeh *et al.* 1998). A second is cell surface interaction which results from specific functional groups on the cell wall that bind Cd or exclude it (Horitsu *et al.* 1986; Mahler *et al.* 1986; Wang *et al.* 2000). The third is intracellular interactions such as export and intracellular sequestration (Morozzi *et al.* 1993; Rosen 1999). More than one mechanism may be employed by the same organism depending on the strain and the growth conditions employed (Roane & Pepper 1999).

Laboratory scale microbial and toxic metal interaction studies require an understanding of how Cd behaves in the medium being used and how that medium relates to environmental conditions (Jonas *et al.* 1984). Only when abiotic interferences are eliminated or accounted for, can it be concluded that the transformation of the toxic metal is the result of interactions with microorganisms or their metabolites. In this study cultures were enriched in minimal media developed specifically for Cd stress studies with microbial consortia.

## Materials and methods

### *Media preparation*

Culture media were prepared anaerobically by the modified Hungate method (Hungate 1969; Miller & Wolin 1974) in 100 ml vials and transferred to 25-ml Balch tubes (Balch & Wolfe 1976). Glucose, vitamin solution, iron supplement, and Cd were added as anaerobic sterile solutions after autoclaving. Cd, added as $CdCl_2$, remained soluble as confirmed by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) analysis (Robinson 1994). Physiological conditions were varied by providing different electron donors and acceptors (d/a). They included: MET (d/a: acetate/CO2) (Oremland 1988), SRB (d/a: lactate/sulfate) (Widdel 1988), FRM (d/a: glucose/no electron acceptor) (Stumm 1988), and multiphysiological (MPH) (d/a: glucose/sulfate) (Stumm 1988; Widdel 1988). Basal media components were (g/l): 0.5 citric acid, 0.1 $MgSO_4 \cdot 7H_2O$ (0.08 $MgCl_2 \cdot 6 H_2O$ in FRM), 1.0 $(NH_4)_2SO_4$ (0.8 $NH_4Cl$ in FRM), 0.0006 ferrous sulfate$\cdot 7H_2O$ (0.0005 ferric citrate in FRM), 0.03 $CaCl_2 \cdot 2H_2O$, 1.0 HEPES, 0.212 glycerol 2-phosphate, 1.0 ascorbic acid, electron donors (added as listed above) 2.3 glucose, 6.0 sodium lactate 60% (w/v), 1.32 sodium acetate. Head space components were added aseptically after inoculation. Vitamin solution was added per Demain & Solomon (1986).

Growth of each physiological type in each medium was confirmed by standard MPN techniques in cultures without Cd. The presence of SRBs were confirmed by ASTM Method D4412-84 (SRB-A) (Eaton *et al.* 1995). Fermenting organisms were demonstrated by the production of acid in a medium containing 3 g beef extract, 5 g peptone, 5 g glucose, 0.5 g sodium thioglycolate, 0.7 g agar, 0.25 g L-cysteine, and 1 g of bromocresol purple added to 1 l of DI water (F-A). Methanogenesis (M-A) was confirmed by the presence of methane in the culture headspace when analyzed by Gas Chromatograph with Flame Ionization Detection. Media were inoculated (2% v/v) with fresh anaerobic digester sludge from the Moscow, Idaho sewage treatment plant. Initial inoculation cultures were allowed to incubate for one day then subcultured into a second set of media. Subculturing was performed when an absorbance of 0.2 OD600nm or the apparent maximum was reached.

### *Post-growth analysis*

Cultures were analyzed for Cd remaining in solution and sulfide concentrations. Samples were collected by syringe and filtered through a 0.2 $\mu$m syringe filter. Cadmium was analyzed by ICP-AES. Sulfide concentration was analyzed by the methylene blue procedure (Vogel 1989).

### *Enrichment*

The inoculation scheme for analysis of consortia development and siderophore interaction is shown in Figure 1. Test 1 was the control condition with no Cd; Test 2 was subcultured with constant Cd level; Test 3 was subcultured with constant Cd concentration and iron (Fe); Test 4 was subcultured with increasing Cd concentration and Fe. The tests were designed

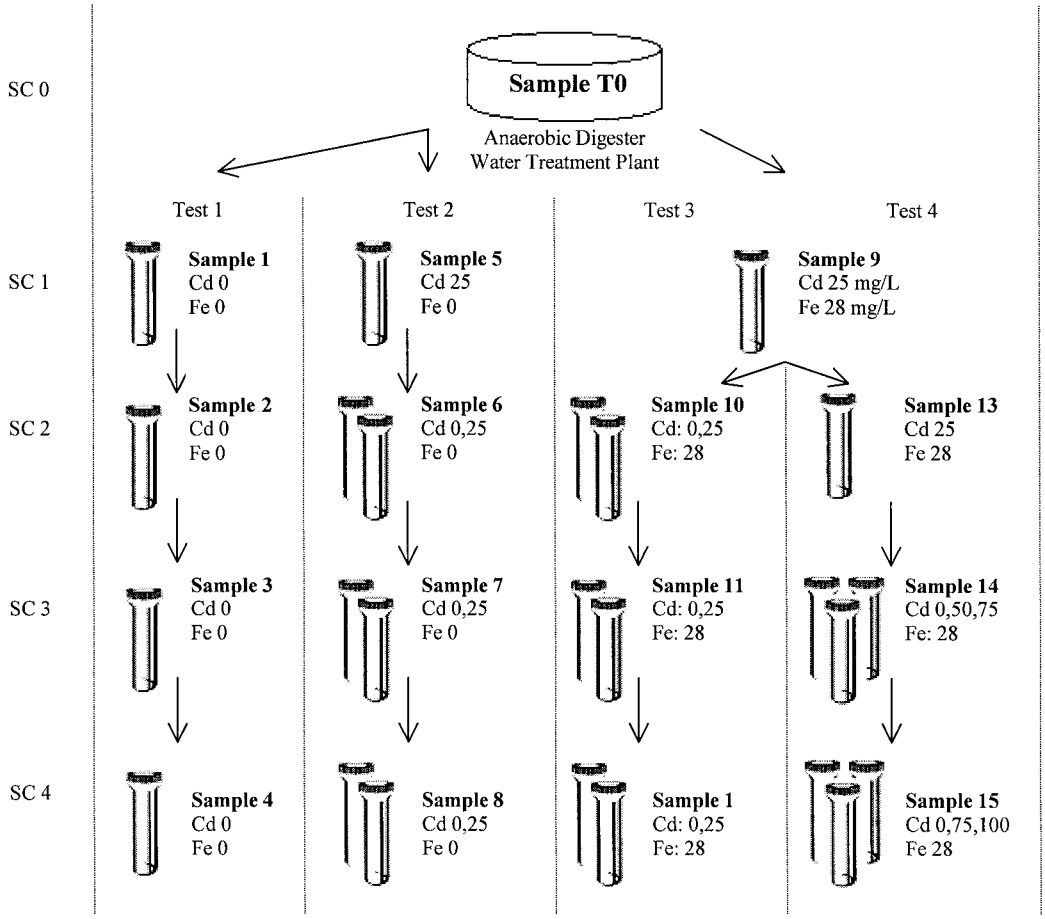


*Fig. 1.* Inoculation scheme for enrichment of Cd resistant consortia, sample numbers correspond to rRNA sample identification number, each succesive inoculation was referred to as a subculture [Cd (mg/l), Fe (mg/l), SC=subculture].

to determine if consortia development was primarily effected by media components, Cd stress, or Fe supplementation/siderophore formation. Samples for analysis of culture development were taken at the time of subculture inoculation.

The inoculation scheme used for Cd resistance development, lag phase comparison, and chelation analysis was similar to Tests 1 and 4 subcultured from SC0 through SC2 (Figure 1). However, there was no Fe supplementation. The chelation analysis was run in MPH media without citrate or HEPES in SC0 and then with and without citrate and HEPES in SC1 for absorbance data point collection.

*DNA analysis*

Total DNA was isolated from 1.5 ml of mixed bacterial cultures or isolates by a modified method of Purdy *et al.* (1996) The cells were pelleted by centrifugation and resuspended in 0.5 ml cell suspension buffer (120 mmole/l $NaPO_4$ buffer, pH 8, 1% polyvinyl polypyrrolidone). Samples were agitated twice in a Bead beater for 2 min at maximum speed and 4 °C with 0.5 g $ZrO_2/SiO_2$ beads, 35 $\mu$l 20% SDS, and 0.5 ml phenol, pH 8.0. Aqueous phase was separated by centrifugation and the samples were loaded onto a hydroxyapatite spin-column equilibrated with 120 mmole/l sodium phosphate buffer, pH 7.2 and then subjected to centrifugation at 120 $\times$ *g* for 2 min. After three washing steps with 0.5 ml 120 mmole/l sodium phosphate buffer, the nucleic acids were eluted with 0.4 ml 1 mole/l potassium phosphate buffer, pH 7.2. The DNA was desalted on a water saturated Sephadex G-50 column. Sample volume was reduced by vacuum centrifugation.

The 16S rDNA was amplified using primers 907R and GC clamp-GM5F, which includes a 5′ 40 base GC clamp (Muyzer *et al.* 1993). Conditions were

0.2 mmole/l dNTP, 1.5 mmole/l $MgCl_2$, 0.5 $\mu$mole/l primers, 1% Tween-20, 5% DMSO, 1–5 $\mu$l template DNA and 1–2 units *Taq* polymerase at 50 $\mu$l volume. Samples were denatured by heating to 95 °C for 5 min. Amplification was accomplished using the 'touch down' program to reduce amplification of non-target products. This program consists of an annealing temperature of 65 °C that is decreased by 0.5 °C each cycle for 20 cycles, an elongation temperature of 72 °C held for 30 sec, followed by denaturation at 95 °C. Twenty cycles of amplification are run at the final annealing temperature with the elongation period extended by 2 sec each cycle. Final extension is run for 5 min at 72 °C. Samples without template and with standard organism mixtures were run to verify accuracy of the method.

DGGE was conducted on a 6% acrylamide/biacrylamide gel (19:1) with a 20–60% denaturing gradient (denaturant composed of 7 mole/l urea, 40% formamide) on a Bio-Rad DGGE apparatus. The 10 $\mu$l of PCR product were diluted with an equal volume of loading buffer (70% glycerol, 2 mmole/L EDTA, pH 8, 0.05% Xylene Cyanol, 0.05% Bromophenol blue) and subjected to electrophoresis at 60 °C and 200 V for 4 h. The DNA was visualized using Sybr green I.

*Isotherm study*

The effect of the microbial growth on the binding of Cd to soil was examined with a langmuir isotherm study. The study was run with three separate tests each with 2.5, 5.0, 7.5, 10, 20, and 30 mg of soil. The first test was a Control run with sterile soil. The second was an Acid test run with sterile soil and pH adjusted to 5.0 with HCl. The third was a Growth run with non-sterilized soil. The soil was sterilized with chloroform in a glass desiccator under vacuum for one month. The chloroform treatment was followed by autoclaving, incubation at 37 °C for one week and then autoclaving again. All tests were run in triplicate in 100 ml crimp top vials with MPH media as described earlier with 2 times the glucose. Cultures were incubated for three weeks at 37 °C. Samples were collected using a sterile syringe and 0.45 $\mu$m filter. The filtered samples were analyzed for soluble Cd by ICP-AES.

## Results and discussion

*Media development*

Understanding and controlling abiotic interactions of toxic metals with culture media components is indispensable when studying metal toxicity in laboratory cultures. It is important to study these interactions on a case by case basis, as a change in environmental conditions can change the resistance mechanism employed by microbial populations (Roane & Pepper 1999) and the actual level of bioavailable Cd (Jonas *et al.* 1984). Initial studies of Cd toxicity to anaerobic bacterial consortia were run in Starkey's Medium C (Table 1) (ATCC 1992). These tests showed that the Cd in the medium had little or no effect on growth of cultures (unpublished data). However, screening tests of time zero samples indicated that there were numerous interactions of Cd with media components and the sludge inoculum. A significant amount of the Cd (86–87%) precipitated abiotically in the pH range of 6 to 9.

The development of a medium for studying Cd toxicity began with a medium used previously for aerobes, and had little or no interactions with added Cd (Roane & Kellogg 1996). The medium, Medium 1 (Table 1), was prepared anaerobically using a modified Hungate technique. Initially, only reductant, buffer, oxygen indicator, and sodium lactate, were added to the published medium formulation.

The effects of pH, reductant levels, and inoculum levels were evaluated in the enrichment screening. Initial pH was a significant variable with an optimum of 6.8 for MPH, 7.0 for FRM, 7.2 for MET, and 6.8 for SRB. Ascorbic acid, used as the reductant, above 0.1% (w/w) did not increase growth, and sludge inoculum levels of less than 2% (v/v) did not result in adequate growth. The carbon source requirement was also significantly higher than would be expected in an aerobically-incubated medium, as was expected for anaerobes based on previous literature (Roane & Kellogg 1996; Tanner 1996). Medium 1 was modified based on these initial findings, to create Medium 2. This medium did not form the typical black precipitate of iron sulfide present in most SRB cultures. Instead a yellow precipitate was formed, which was assumed to be cadmium sulfide (CdS). Comparison of the turbidity of the cultures indicated that there was no increase in stress in cultures between 50 mg/l and 90 mg/l Cd. Interaction of Cd with inorganic phosphate ($Na_4O_7P_2$) was suspected; therefore, it was replaced with glycerol

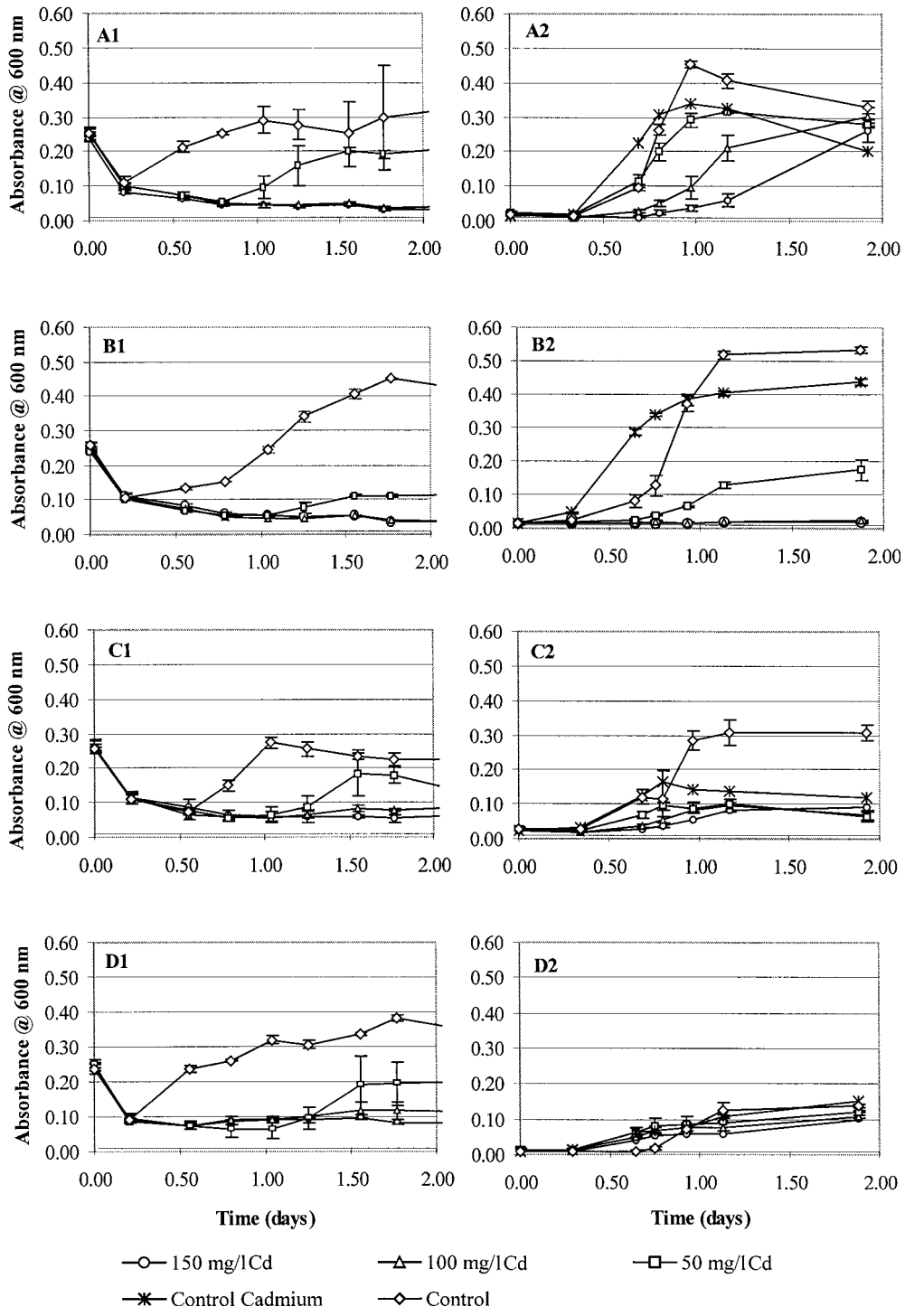


*Fig. 2.* Cd stress is indicated by lower growth in higher Cd concentrations. Adaptions is indicated by a shorter lag time in comparison of subculture 1 to subculture 2 of Cd containing enrichments. (A. MPH B. SRB C. MTH D. FRM. Numbers refer to subculture).

*Table 1.* Recipes for media throughout development phase and the percentage of Cd left in solution. Media was developed to ensure bioavailable Cd.

| Component | | Starkey's | Medium 1 | Medium 2 | Final medium |
|---|---|---|---|---|---|
| NTA trisodium salt | | 0.2 g/l | | | |
| Citric Acid | | | 0.5 g/l | 0.5 g/l | 0.5 g/l |
| Ferric Citrate | | | | 0.0006 g/l | |
| $MgSO_4 \cdot 7H_2O$ | | 2 g/l | 0.1 g/l | 0.1 g/l | 0.1 g/l |
| $Na_2SO_4$ | | 1 g/l | | | |
| $(NH_4)_2SO_4$ | | | 1.0 g/l | 1.0 g/l | 1.0 g/l |
| Ferrous Sulfate | | | | | 0.0006 g/l |
| $CaCl_2 \cdot 2H_2O$ | | 0.1 g/l | | 0.03 g/l | 0.03 g/l |
| $NH_4Cl$ | | 1 g/l | | | |
| $KH_2PO_4$ | | 0.5 g/l | | | |
| Pyrophosphate tetra sodium | | | .017 g/l | .017 g/l | |
| Glycerol 2-Phosphate | | | | | 0.212 g/l |
| Yeast Extract | | 1 g/l | | | |
| Vitamin Solution | | | | | Methods section |
| 60% Sodium Lactate | | 5.8 g/l | 3.0 g/l | 3.0 g/l | 3.0 g/l |
| pH adjusted with | | NaOh | NaOh | KOH | KOH |
| HEPES buffer | | | 1.0 g/l | 1.0 g/l | 1.0 g/l |
| Cysteine HCl | | 0.75 g/l | | | |
| Ferrous Ammonium Sulfate | | 0.025 g/l | | | |
| Ascorbic acid | | | 1.0 g/l | 1.0 g/l | 1.0 g/l |
| Soluble Cd | 50 mg/l | 88% ± .018 | 87% ± .012 | 82% ± .003 | 90% ± .008 |
| | 100 mg/l | 86% ± .006 | 90% ± .001 | 72% ± .001 | 97% ± .003 |

pyrophosphate, an organic form ($C_3H_9O_6P$) to create the final medium (Trevors *et al.* 1985; Roane & Kellogg 1996). The turbidity of the cultures grown in the final medium indicated that the stress was increasing with Cd concentration (Figure 2B1). This media was varied by changing the electron donors and acceptors to produce differing enrichment conditions. The results of the physiological type confirmation are shown in Table 2. The amount of Cd in solution at time zero was $94 \pm 2\%$ for all media. The amount of Cd left in solution after culture growth was: $87 \pm 14\%$ for FRM, $84 \pm 14\%$ for MPH, $62 \pm 19\%$ for MET, and $55 \pm 11\%$ for SRB.

Enrichment experiments in the Final Medium indicated that there was Cd stress under all physiological conditions. As shown in Figure 2 growth decreased as Cd concentration was increased, indicating that Cd in the medium was bioavailable. This was true for all enrichment conditions in both subculture 1 (SC1) and subculture 2 (SC2) with the exception of the FRM run on SC2. This particular inoculation run did not have strong growth. This was due to the inoculant sludge variability. Experiments run under the same conditions with sludge taken from the water treatment plant reactor at differing times showed differing results for FRM enrichments. In some of the runs the FRM enrichments showed one of the highest resistance levels after MPH. The FRM run in Figure 2 is shown for consistency, since it was inoculated with the same sludge sample as the other enrichments shown in Figure 2.

*Table 2.* Phsyiological type confirmation in media enrichments, media was varied by providing differing electron acceptors and donors (+ positive test result, − negative test result.

| Medium | SRB-A | F-A | M-A |
|---|---|---|---|
| FRM | − | + | − |
| MPH | − | + | − |
| MET | + | Not run | + |
| SRB | + | + | + |

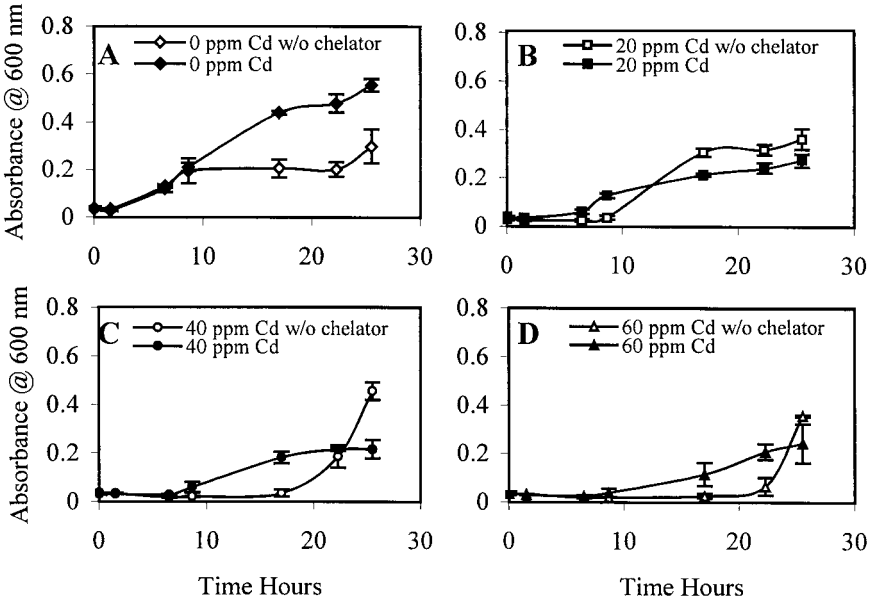


*Fig. 3.* Effects of chelators on Cd toxicity. Chelators provide a transient decrease in toxicity.

*Chelation analysis*

A separate test was run to evaluate the effects of chelators on Cd toxicity in the MPH medium. Chelators bind metals and are able to keep them in solution. Some chelators will bind metals in a non-bioavailable form (Malakul *et al.* 1998; Pazirandeh *et al.* 1998) causing a false resistance mechanism to be deduced. However, the chelation molecule may be transformed so that it releases the metal ion (Thomas *et al.* 1998). As shown in Figure 3, the presence of citrate and HEPES effected growth under control and stress conditions. In controls, Figure 3A, these compounds increased overall growth. The effect may have been due to the use of citrate as a carbon source or the HEPES as a buffer. Cultures with Cd and chelators, Figures 3B, 3C, and 3D, had a transient decrease in Cd toxicity; however, growth of the cultures without the chelators recovered. It was also determined in these cultures that the Cd was ultimately released from the chelation molecules, as shown by ion selective probe that does not detect Cd if bound to another molecule. This finding was supported by Electrospray Ionization Mass Spectrometry (ESI-MS) analysis of a fraction of the supernatant separated on Bio-Rad P2 gel (data not shown). A Cd signature was not found in the ESI-MS spectrum. This indicates that the Cd was most likely free in solution and could not be ionized. The presence of Cd in this fraction was verified by ICP-AES.

The level of Cd resistance of the consortia, as shown by an increase in overall growth at higher Cd levels and decreased lag phase upon subculture, increased most significantly for the MPH culture conditions. A comparison of overall growth is shown in Figure 2. Comparison of MPH SC1 vs. MPH SC2 indicates that the cultures grown at 100 and 150 mg/l Cd had a significant increase in resistance. A similar comparison of the other three enrichment conditions shows no significant increase and even a decrease in growth under the SRB conditions at 100 and 150 mg/l Cd. This may be due to a dilution of the original inoculant containing trace nutrients and/or Cd binding compounds. A lag phase comparison is shown in Figure 4. In this figure the solid lines indicate the cultures containing Cd. All cultures except the FRM culture showed a decrease in lag phase at the 50 mg/l Cd levels.

*DNA analysis*

The 16S rRNA profiles showed that the consortia underwent succession under all physiological conditions when compared to the profile of the inoculum. Succession stabilized by the fourth cycle of subculturing. Under SRB conditions (not shown), the presence of Cd led to four predominant bands in the DGGE analysis independent of the iron concentration. In the control (no Cd) only one predominant band emerge after four subculturing cycles. Cd resistant organisms

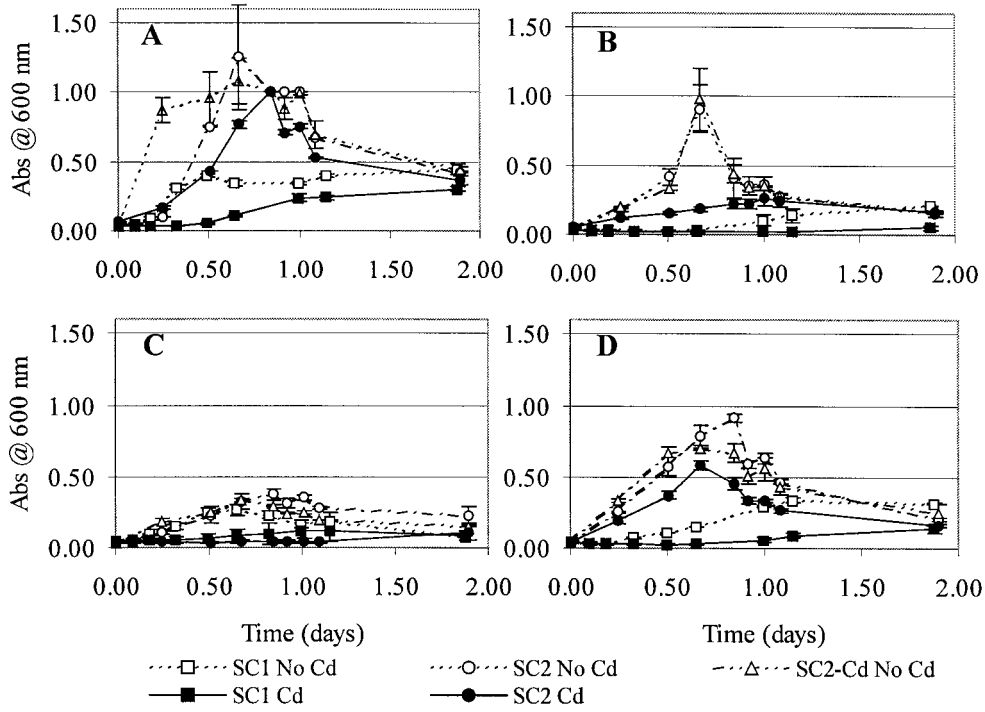


*Fig. 4.* Lag Phase Comparison of subculture 1 and subculture 2: A. MPH - significant reduction in lag phase. B. MET – a slight reduction in lag phase. C. FRM – insignificant growth, this was not typical of all FRM enrichments D. SRB – significant reduction in lag phase (SC subculture).

enriched under SRB conditions include *Veillonella* sp., *Klebsiella* sp., and *E. coli.*, based on 16S rRNA gene sequencing and comparison of the sequences to the Basic Local Alignment Search Tool (BLAST®) (National Institute of Health 2002).

Culture development under MPH conditions is shown in Figure 5(A). Under these culture conditions the medium was not the primary selection influence, as shown by the differences in band development in lanes 1–3. The addition of iron (lanes 9–12), allowed a variety of species to prosper in addition to the species selected without iron enrichment (lanes 5–8). These lanes also indicate that resistance was not due simply to enrichment of siderophore producers, by the presence of the same organism with and without Fe. Cd resistant organisms enriched under MPH conditions included *Klebsiella* sp., and *Enterbacter* sp. As shown in Figure 5A, *Enterbacter* (1B) was isolated from the MPH culture, though it was not a dominant member in the enrichment. It should be noted that the organisms enriched in from Test 1 (the no Cd control) were able to tolerate Cd at low concentrations in all but the MET consortia. This indicates that low level Cd resistance was intrinsic in these organisms and implies that the resistance seemed to be due to fortuitous selection of the media. FRM conditions in Figure 5B selected for the same species as the control (lanes 1–4), Cd-containing (lanes 5–8), and increasing Cd with iron (lanes 9–10). This shows that culture adaptation in this FRM enrichment was due primarily to medium formulation rather than Cd stress. It should be noted that these enrichments are not the same as those discussed in Figures 2 and 4.

### *Cadmium resistance mechanism of isolate*

*Klebsiella* sp., which was isolated from the MPH enrichment, was the only isolate that could be maintained with its cadmium resistance remaining significantly active. This isolate was further studied to identify its Cd resistance mechanism. *Klebsiella* species have been found to precipitate Cd (Christensen 1985) or exclude the Cd from the cell by producing an extracellular capsule (Bitton & Freihofer 1978). Under our enrichment conditions, however, the *Klebsiella* was found to leave the Cd in solution, and it was able to tolerate concentrations up to 250 mg/L soluble Cd. Sulfide was found in the cultures at a concentration of 0.96 mg/l sulfide and 0.34 mg/l sulfide in MPH growth media with 100 mg/l of 100 mg/l Cd and 146.6 mg/l of 150 mg/l added Cd remaining in solution, respec-

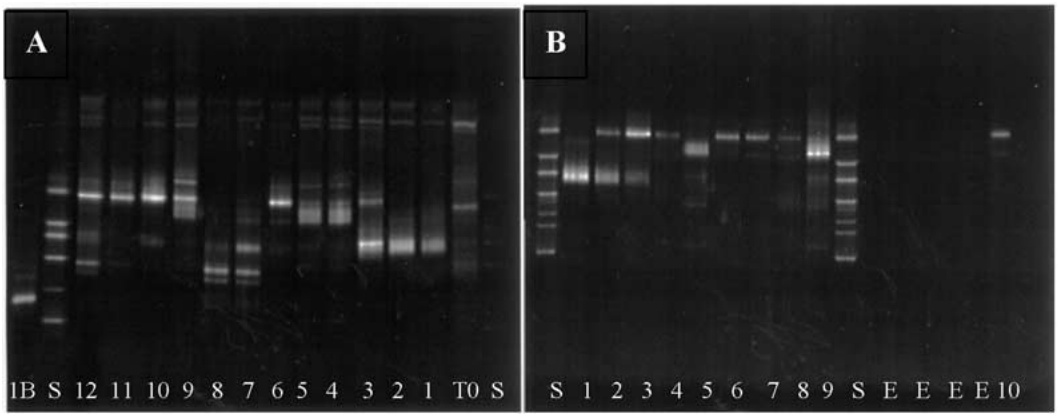


*Fig. 5.* (A) MPH enrichments indicate culture development. (B) FRM enrichments appear to be enrich for the same organism under all culture conditions, indicating media selection rather thand cadmium resistance development. [Lane numbers indicate sample number (Figure 1) S−standard, E=empty, 1B=isolate *Enterbacter sp.*]

tively. We suspected that the organism was producing a compound that was chelating or transiently binding the Cd, thereby reducing Cd bioavailability. There have been studies that have documented the use of siderophores or chelating adsorbents (Malakul *et al.* 1998) in metal detoxification. Clark (Clarke *et al.* 1987) found that siderophore activity moderated copper toxicity, however the production of the siderophore was not in response to the metal toxicity. Lawford found an anionic polymer that sequestered and stored Zinc (Lawford *et al.* 1980). Chanmagathas and Bollag found a low molecular weight Cd binding molecule that could not be identified but was determined not to be a metallothionein (Chanmagathas & Bollag 1998). In contrast a Cd binding protein with a molecular weight of approximately 39,000 has also been reported (Mitra 1984).

A lyophilized supernatant sample of a *Klebsiella* sp. grown in MPH media was resolubilized in DI and chromatographed on a P2 size exclusion column. Fractions were then collected and assayed using ICP-AES to determine Cd presence. The Cd-containing fraction was run on ESI-MS. No Cd signature found, implying that the Cd was free in solution. In addition, the Cd-containing fraction was evaluated by free Cd ion selective probe, which showed free Cd was present. It was therefore concluded that during the growth of this Klebsiella a Cd binding molecule was only transiently binding the Cd or else was non-existent, because the Cd was detected by the ion selective probe.

*Isotherm study*

In order for laboratory experiments to be relevant in the environment the experimental design must adequately mimic the actual environment (Jonas *et al.* 1984). Throughout these experiments we found that the MPH medium consistently produced the highest Cd resistance while leaving the Cd in solution. We hypothesized that this medium could be used in a mixed waste environment to potentially biodegrade a co-contaminant prior to removing the Cd contamination by another removal method.

Leachate from municipal compost, waste incinerator slag, and sewage sludge results in reduced soil sorption due to increased ionic strength, inorganic and organic ligands, competing cations such as zinc, and organics, which can coat surfaces or pull Cd into solution. The presence of waste leachates, as compared to unpolluted soil solution, decreases the distribution coefficients 30 to 250 times. As a result the relative migration velocity of Cd present in waste leachate is 80–170 times faster for the same pH in an unpolluted soil solution (Christensen 1985).

Cd distribution coefficients are reduced 2 to 14 times due to competition with other metals. Mixtures of nickel, cobalt, and zinc or chromium, copper, and lead, reduced Cd sorption onto soils, and zinc accounted for most of the observed competition with Cd, perhaps due to the comparable chemistry and that Zn concentrations in the soil are usually 100 to 1000 times higher than Cd concentrations (Christensen 1987). To test the effects of MPH medium on the Cd sorption capacity of soil a Langmuir isotherm test was used to evaluate if the Cd would stay in solution. Cd tends

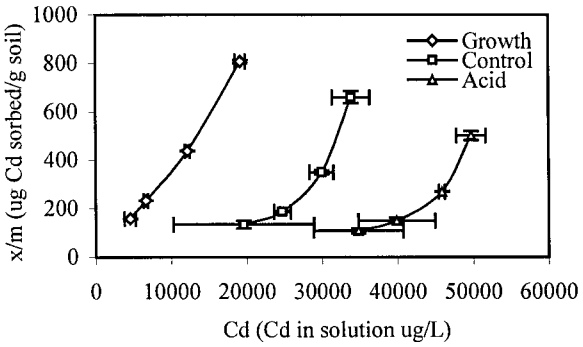


*Fig. 6.* Langmuir Isotherm: Cd sorption was the lowest in the acid test, followed by the control test, and was the highest in the growth test.

to bind to organics in soils, which makes the Cd non-bioavailable, but the Cd is left *in situ*, which makes it available for transport into ground water if chemical or microbiological conditions change appropriately. Sorption capacities decrease with decreasing pH (Christensen 1984). This is of great concern, since the acidification of soils by acid rain or microbial activity can mobilize Cd and make it bioavailable (Palm 1994). In previous work, it was found that as much as 36% of Cd sorbed to soil (20 mg Cd/70 g soil) was mobilized in the presence of microorganisms and nutrients, while less than 16% was released from sterile and unsupplemented soil controls (Chanmagathas & Bollag 1987).

We tested to determine if the soil Cd solubility was due to acidic conditions or an additional compound that was binding the Cd (Chanmagathas & Bollag 1987, 1998). It was expected that there was transient chelation with the metabolites in the growth cultures. If this was correct the amount of Cd left in solution would be lowest in the sterile control, higher in the sterile control with pH adjustment to pH 5, and highest in the growth culture. The control and acid results responded as expected relative to one another (Figure 6), however the growth experimental results gave a very different result. The sorption of the Cd to the soil was found to be higher than the control experiment. While this indicates that the medium did not increase the solubility of the Cd it should be noted that a biomass control was not conducted. This variable would need to be controlled to determine if the medium was decreasing the sorption of the Cd to the soil. The amount of Cd left in solution may be too low for this information to be relevant and further tests may not be significant unless bioaugmentation was attempted with one of the isolates, such as *Klebsiella pneumonia*.

## Conclusions

This work describes development of a medium designed specifically for anaerobic microbial Cd stress studies. In this medium Cd toxicity was initially present under all physiological conditions tested. Cd resistance increased with sub-culturing for all physiological conditions. However, the more effective resistance mechanisms seemed to be due to fortuitous selection. In fact, some physiological conditions, especially those that left the Cd free in solution, seemed to be dependent on the enrichment type rather than Cd selection as demonstrated by the development of Cd resistance in control cultures. This work also demonstrated that development of Cd toxicity resistance does not necessarily require that the metal be removed from solution and that the resistance mechanism used by specific microbes may be dependent on medium formulation in which they are grown. The results also show that anaerobic microorganisms can become Cd resistant while leaving Cd in solution. Future work is required to determine if mechanisms such as Cd efflux or other resistance mechanisms are involved.

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