Carboxymethyl-β-cyclodextrin mitigates toxicity of cadmium, cobalt, and copper during naphthalene biodegradation

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ABSTRACT

Hazardous waste sites are commonly contaminated with both organic and metal pollutants. Many metal pollutants have been shown to inhibit organic pollutant biodegradation. We investigated the ability of a modified, polydentate cyclodextrin (carboxymethyl-β-cyclodextrin, CMCD) to reduce the toxicity of 33.4 μM cadmium, cobalt or copper during naphthalene degradation by a *Burkholderia* sp. in 120 h aerobic, batch studies. The highest investigated concentration of CMCD, 3340 μM, reduced cadmium, cobalt, and copper toxicity. With each metal, the length of the lag phase was reduced (by as much as 108 h with cobalt or copper), the cell yield was increased (by as much as a factor of 16 with cobalt), and the growth rate was increased (by as much as a factor of 31 with cobalt). The degrader was unable to use CMCD as the sole source of carbon and energy. Our data suggest that the ability of CMCD to complex metals plays an important role in its ability to mitigate metal toxicity and that CMCD has the potential to enhance biodegradation in organic and metal co-contaminated environments.

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1. Introduction

Hazardous waste sites on the US Environmental Protection Agency's (EPA) National Priority List (NPL) are frequently contaminated with both organic and metal pollutants that pose health hazards to humans and wildlife (Sandrin and Hoffman, 2007). The low aqueous solubility and bioavailability of many of the organic pollutants can limit the efficacy of biologically-based strategies for their removal (i.e., bioremediation). In metal and organic co-contaminated environments, common metals include arsenic, cadmium, chromium, copper, lead, mercury, nickel, and zinc that have been found to be toxic to many biodegrading microorganisms (Amor et al., 2001; Benka-Coker and Ekundayo, 1998; Degraffenreid and Shreve, 1998; Riis et al., 2002; Roane et al., 2001; Springael et al., 1993; White and Knowles, 2000, 2003). Thus, effective bioremediation-based strategies used in metal and organic co-contaminated environments must address two key issues: (1) limited organic pollutant solubility and bioavailability and (2) toxic metals that inhibit biodegrading microorganisms.

Several strategies to limit metal-inhibition of organic pollutant biodegradation have been proposed and include use of: cadmium-sequestering soil microorganisms (Roane et al., 2001), calcium carbonate (Jonioh et al., 1999), chelating agents such as EDTA (e.g., Spencer and Nichols, 1983), and surfactant-modified clays (Malakul et al., 1998). Each of these strategies addressed only one of the two key issues limiting biodegradation in co-contaminated environments by focusing only on reducing bioavailable metal concentrations. In contrast, Sandrin et al. (2000) employed a biological surfactant, rhamnolipid, to enhance naphthalene biodegradation in the presence of cadmium. Rhamnolipid has been shown to bind metals such as cadmium (Tan, 1994) and enhance organic pollutant solubility, thus allowing it to address both impediments to biodegradation. Results were encouraging: a 10-fold greater concentration of rhamnolipid than cadmium allowed enhanced naphthalene biodegradation. These results suggest that strategies that address both impediments to biodegradation have promise. Rhamnolipid is a monodentate molecule (i.e., has a single metal-complexation site). The use of multidentate, metal-complexing agents capable of increasing organic pollutant solubility and bioavailability to enhance biodegradation in co-contaminated systems has not been investigated.

Cyclodextrins are cyclic polymers of α-D-glucopyranoside units that have been shown to enhance the aqueous solubility of many hydrocarbons including anthracene, pentachlorophenol, phenanthrene, naphthalene, and toluene (Badr et al., 2004; Furuta et al., 2007; Hanna et al., 2004; Horsky and Walterova, 2005; Lubomska et al., 2005; McCray, 2000). Such enhancements to aqueous solubility seem to allow concomitant increases in hydrocarbon bioavailability, removal, and biodegradation (Bardi et al., 2000; Ramsay et al., 2005; Steffan et al., 2002).

Cyclodextrins can be modified by the addition of various substituents. For example, carboxymethyl groups are added to the hydroxyl groups of the β-cyclodextrin core structure to form carboxymethyl-β-cyclodextrin (CMCD). This cyclodextrin derivative has been demonstrated not only to increase the aqueous solubility of anthracene, trichlorobenzene, biphenyl, and DDT, but also to complex cadmium simultaneously (Wang and Brusseau, 1995). In addition, CMCD has been shown to complex nickel and strontium (Brusseau et al., 1997). Given the demonstrated abilities of CMCD to increase organic pollutant bioavailability and to complex metal pollutants, CMCD may be especially effective in enhancing the biological removal (i.e., bioremediation) of organic pollutants in metal and organic co-contaminated environments.

The objective of this study was to examine the ability of CMCD to enhance organic pollutant biodegradation by reducing metal toxicity. To accomplish this, we employed a model system consisting of cadmium, cobalt and copper as model metal pollutants and naphthalene as a model organic pollutant. The ability of CMCD to reduce metal-inhibition of naphthalene biodegradation by a model degrader, a naphthalene-degrading *Burkholderia* sp., was assessed in batch solution studies. CMCD reduced inhibition associated with all three metals. Our results suggest that CMCD may be useful in increasing biodegradation in metal and organic co-contaminated environments and that the ability of CMCD to complex metals accounts, in part, for its ability to reduce metal toxicity.

2. Methods

2.1. Model microorganism, microbiological media, and cyclodextrins

A naphthalene-degrading *Burkholderia* sp. was isolated and identified as previously described (Sandrin et al., 2000). Culture medium and stock solutions were prepared using water from a Culligan Hi-Flo® 42 Water Filter with Cullar® Carbon Media (USFilter, Warrendale, PA) with a resistance of 18.2 MΩ. A minimal salts medium (MSM) was used and consisted of the following components (g L^{-1}): KH$_2$PO$_4$ (0.1), Na$_2$HPO$_4$ (0.1), NH$_4$NO$_3$ (0.05), (NH$_4$)$_2$SO$_4$ (0.05), MgSO$_4$ (0.02), CaCl$_2$ (0.002), MnSO$_4$ (0.0002), and FeCl$_3$ (0.0002). The solution was passed through Fisherbrand® P8 grade cellulose filter paper (Fisher Scientific, Pittsburgh, PA) to remove suspended solids, and the pH of the medium was adjusted to 6.5–7.0 prior to sterilization via autoclaving.

Technical grade CMCD (Cerestar Cavitron 822N4; lot no. 1Y0031) was the kind gift of Cargill, Inc. (Minneapolis, MN). The purity of the CMCD was not reported, nor was the average degree of substitution. Unmodified β-cyclodextrin has a molecular weight of 1135 amu. Assuming a degree of substitution of 3.5 carboxymethyl substituents (each with a molecular weight of 58 amu) per CMCD molecule, we calculated an average atomic mass for CMCD of 1338 amu. This molecular weight was used to calculate the requisite masses of CMCD to construct solutions with the desired concentrations. A cyclodextrin derivative with no known metal-binding capacity, hydroxypropyl-β-cyclodextrin (HPCD), was the kind gift of Wacker Chemical Corporation (Munich, Germany). An average atomic mass for HPCD of 1193 amu was used to calculate the requisite masses of HPCD to construct solutions with the

desired HPCD concentrations. Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) (Bruker Reflex IV; Bruker Daltonics, Billerica, MA) was used qualitatively to verify the presence of carboxymethyl and hydroxypropyl substituents on the cyclodextrin core structures.

2.2. Metal sensitivity assays

Sensitivity to cadmium, cobalt, or copper was determined in pure culture, batch growth studies. Cells from a 16–20 h pre-culture (containing $\sim 10^8$ CFU mL^{-1}) grown at 25 °C with shaking (200 rpm) were diluted 1:10^4 into 125 mL Nalgene® flasks containing 50 mL sterile MSM. Naphthalene (300 mg L^{-1}) was added as the sole source of carbon and energy in excess of its aqueous solubility (30 mg L^{-1}). CMCD or HPCD was added at concentrations of 0, 33.4, 334, and 3340 μM. Cadmium, cobalt, and copper were added as cadmium nitrate (Cd(NO$_3$)$_2$·4H$_2$O), cobalt chloride (CoCl$_2$·6H$_2$O), and copper chloride (CuCl$_2$·2H$_2$O) to bring total metal concentrations in triplicate flasks to 33.4 μM. Cells (0.5 mL from the 10^8 CFU mL^{-1} pre-culture) were added to flasks containing media within one minute of adding the metal. Nitrate concentrations were equalized in flasks containing cadmium using KNO$_3$. All cultures were incubated on an orbital shaker (200 rpm) at 25 °C. Culture turbidity (OD$_{540}$) was determined periodically and used as the measure of growth to construct growth curves and to assess metal effects on biodegradation as previously described (Hoffman et al., 2005; Malakul et al., 1998; Sandrin et al., 2000; White and Knowles, 2003). Periodically, samples (1 mL) were obtained and centrifuged at 14,000g for 5 min. Supernatants were removed, cell pellets were resuspended in 1 mL of sterile medium, and the OD$_{540}$ of each sample was determined (GENESYS 2 spectrophotometer; Thermospectronic, Woburn, MA). The ability of the degrader to use CMCD as the sole source of carbon and energy was assessed by conducting similar batch growth studies in which flasks were supplemented with CMCD rather than naphthalene.

2.3. Metal complexation

Cadmium was used as a model divalent metal to assess the abilities of CMCD and HPCD to complex metals in our system. An ion-selective electrode (Orion model 9448; Beverly, MA), which measures only free, uncomplexed cadmium, was used according to the manufacturer's instructions to measure cadmium complexation by CMCD and HPCD, except that the use of an ionic strength adjuster (5 M NaNO$_3$) was omitted due to its interference with measurement of complexed cadmium as has been described previously (Sandrin et al., 2000).

2.4. Analysis of data

Three growth parameters were considered to assess metal toxicity during biodegradation of naphthalene: lag phase, cell yield, and growth rate. Lag phase represents the length of time (h) required for the culture to enter a well-defined exponential growth rate. Cell yield represents the maximum optical density (OD$_{540}$) observed in each treatment. Growth rates (OD$_{540}$/h) were calculated using the following relationship: (OD$_f$ – OD$_i$)/t, where OD$_f$ is the OD$_{540}$ at the end of exponential growth, OD$_i$ is the OD$_{540}$ at the onset of exponential growth, and t is represents the length of time (h) of the exponential growth phase. When cultures failed to enter a clearly discernable exponential growth phase, a lag phase length of 120 h (the duration of the growth experiments) was used. In the absence of a clear exponential growth phase, OD$_f$ was represented by the maximum OD$_{540}$ obtained throughout the 120 h duration of the experiment, OD$_i$ was represented by the initial OD$_{540}$, and t was time at which OD$_f$ was obtained.

Table 1
Effect of CMCD on growth kinetics of *Burkholderia* sp. on naphthalene in the absence and presence of 33.4 μM cadmium, cobalt, and copper. Lag phases, yields, or growth rates within the same treatments followed by the same letter are not significantly different as measured by the Least Significant Difference (LSD) test at $P = 0.05$.

[CMCD] (μm)	No metal	Cadmium				Cobalt				Copper			
	0	0	33.4	334	3340	0	33.4	334	3340	0	33.4	334	3340
Lag phase[A] (h)	65.3b	112a	120a	108a	12b	120a	120a	120a	12b	120a	120a	120a	12b
Yield[B]	0.169a	0.030b	0.006b	0.049b	0.367a	0.010b	0.007b	0.041ab	0.157a	0.010b	0.006b	0.012b	0.059a
Log phase growth rate[C] (OD_{540}/h)	0.007a	1.96×10^{-3}b	4.72×10^{-5}b	1.93×10^{-3}b	7.12×10^{-3}a	8.24×10^{-5}b	3.57×10^{-5}b	3.65×10^{-5}b	2.55×10^{-3}a	9.52×10^{-5}b	3.17×10^{-5}b	1.11×10^{-4}b	2.16×10^{-3}a

[A] For cultures that failed to enter a clearly discernable exponential growth phase, a lag phase length of 120 h (the duration of the growth experiment) was used.

[B] Yield represents the maximum OD_{540} obtained over the 120 h course of the experiment.

[C] Growth rates were calculated using the following relationship: $(OD_f - OD_i)/t$, where OD_f is the OD_{540} at the end of exponential growth, OD_i is the OD_{540} at the onset of exponential growth, and t is represents the length (h) of the exponential growth phase. In the absence of a clear exponential growth phase, OD_f represents the maximum OD_{540} obtained throughout the 120 h duration of the experiment, OD_i is the initial OD_{540}, and t is time at which OD_f was obtained.

2.5. Variance analyses

Values for each growth parameter or free ionic cadmium concentration represent averages of three replicates (triplicates). Data were subjected to a univariate Analysis of Variance (ANOVA). A Least Significant Difference (LSD) test was used to identify statistically significant differences ($P \leqslant 0.05$). All statistical calculations were performed using SPSS Statistics software (SPSS, Inc.; Chicago, IL).

3. Results and discussion

3.1. Metal sensitivity

Cadmium, cobalt, and copper, each at 33.4 μM, inhibited growth during naphthalene biodegradation (Table 1; Fig. 1). Biodegradation appeared completely inhibited by cobalt and copper. Cultures containing each metal did not enter log phase growth during the 120 h duration of the experiment, and cell yields were lower in each of the metal-containing treatments than in the metal-free control. In addition, cobalt and copper reduced the growth rate from 0.007 OD_{540}/h in the metal-free control to 8×10^{-5} and 1×10^{-4} OD_{540}/h, respectively.

While there is a paucity of information in the literature on the effects of cobalt and copper on naphthalene biodegradation by bacteria in batch solution studies, reports of inhibition of naphthalene biodegradation by cadmium are relatively common. Malakul et al. (1998) reported that 890 μM cadmium reduced the cell yield of a naphthalene-degrading *Pseudomonas putida* isolate by 50%. We also observed cadmium to reduce the cell yield of the *Burkholderia* sp. isolate we used, but the reduction was greater (82%) with a lower cadmium concentration (33.4 μM). It is possible that the different microbiological media used in each study affected cadmium bioavailability and resulting toxicity differently. Malakul et al. (1998) used tris–HCl as a pH buffer in their experiments; we employed a phosphate buffer. Results of previous studies using phosphate-buffered media (Hoffman et al., 2005; Sandrin et al., 2000; Sandrin and Maier, 2002), are more similar to those in the present study. For example, 100 μM cadmium completely inhibited growth of a naphthalene-degrading *Comamonas testosteroni* isolate (Hoffman et al., 2005). Likewise, only 8.9 μM cadmium was reported to delay exponential growth, and 45 μM completely inhibited growth of the same *Burkholderia* sp. used in the present study (Sandrin et al., 2000).

3.2. Effect of CMCD on growth during naphthalene biodegradation in the presence of metals

The naphthalene degrader was unable to use CMCD as a sole source of carbon and energy (data not shown). CMCD reduced inhibition by all three metals, though to different degrees for each metal (Table 1; Fig. 2A–C). A 100-fold greater concentration of CMCD (3340 μM) than cadmium (33.4 μM) was required to mitigate toxicity. With cadmium (Fig. 2A), this concentration of CMCD reduced the lag phase by 100 h (from 112 h to only 12 h) and increased the cell yield to 0.367 OD_{540} (from 0.030 OD_{540} in the absence of CMCD). Similarly, the growth rate in this CMCD-amended treatment was higher than that observed without CMCD (7.12×10^{-3} OD_{540}/h vs. 1.96×10^{-3} OD_{540}/h).

Similar reductions in cadmium toxicity during biodegradation of naphthalene have been reported with other metal chelating agents. Malakul et al. (1998) reduced cadmium toxicity by 90% in a naphthalene-degrading *P. putida* culture by adding surfactant-modified clay. In the present study, CMCD (3340 μM) mitigated cadmium toxicity in our system to a greater extent than that re-
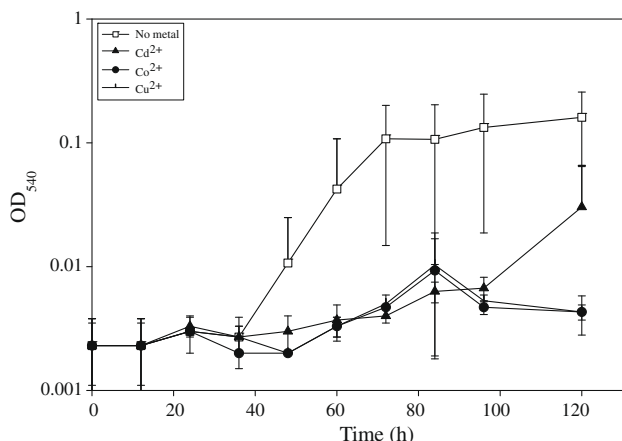
Fig. 1. Effect of 33.4 μM cadmium (Cd^{2+}), cobalt (Co^{2+}), and copper (Cu^{2+}) on growth of a naphthalene-degrading *Burkholderia* sp. Points represent the mean OD_{540} of triplicate flasks. Bars represent one standard deviation above and below each mean.

ported by Malakul et al. (1998): the lag phase was reduced by 89% (from 112 to 12 h), while the cell yield and growth rate were increased by 1223% (from 0.030 to 0.367 OD_{540}) and 363% (from 1.96×10^{-3} to 7.12×10^{-3} OD_{540}/h), respectively. In contrast, CMCD does not appear as efficient at mitigating cadmium toxicity as a rhamnolipid biosurfactant. Sandrin et al. (2000) reported that a 10-fold greater concentration of rhamnolipid than cadmium completely eliminated toxicity. A higher concentration of CMCD (100-fold greater concentration than cadmium) was required to reduce cadmium toxicity comparably.

As with cadmium toxicity, only the highest investigated concentration of CMCD afforded mitigations in cobalt (Table 1; Fig. 2B) and copper (Table 1; Fig. 2C) toxicity. With regard to cobalt toxicity, 3340 μM CMCD reduced the lag phase by 108 h, increased the cell yield increased by 0.147 OD_{540}/h, and increased the growth rate by a factor of 31. Results of experiments with copper were similar. The lag phase was reduced by 108 h, the cell yield was increased 0.049 OD_{540}/h, and the growth rate was increased by a factor of 23.

3.3. Metal complexation

Cadmium was used a model metal to assess the ability of CMCD to complex the metals used in this study and thus reduce their bioavailability. Using a cadmium ion-selective electrode, ionic cadmium concentrations were measured in the microbiological medium containing 0, 33.4, 334, or 3340 μM CMCD. The electrode measures only free Cd^{2+}, the species of cadmium commonly held to be the most bioavailable and toxic (Angle and Chaney, 1989). In the absence of CMCD, 11.9 μM Cd^{2+} was measured. We hypothesized that much of the residual cadmium (33.4 μM total cadmium was added initially) precipitated from the medium as $Cd_3(PO_4)_2$ as has been suggested earlier (Sandrin and Maier, 2002). Addition of CMCD reduced Cd^{2+} concentrations. As CMCD was added at 33.4, 334, and 3340 μM, Cd^{2+} concentrations decreased to 11.3, 8.68, and 4.33 μM, respectively.

While these reductions are significant, they are lower than those reported in a prior study (Sandrin et al., 2000). Addition of a rhamnolipid biosurfactant (890 μM) to the same medium used in the present study reduced Cd^{2+} concentrations to 11.2 μM, representing a 99% reduction. The highest concentration of CMCD in the present study yielded only an 87% reduction. These results may be explained by considering the conditional stability constants, K, for rhamnolipid and CMCD. The stability constant, log K (L/mol), for rhamnolipid is 6.89 (Ochoa-Loza, 2001), while the va-
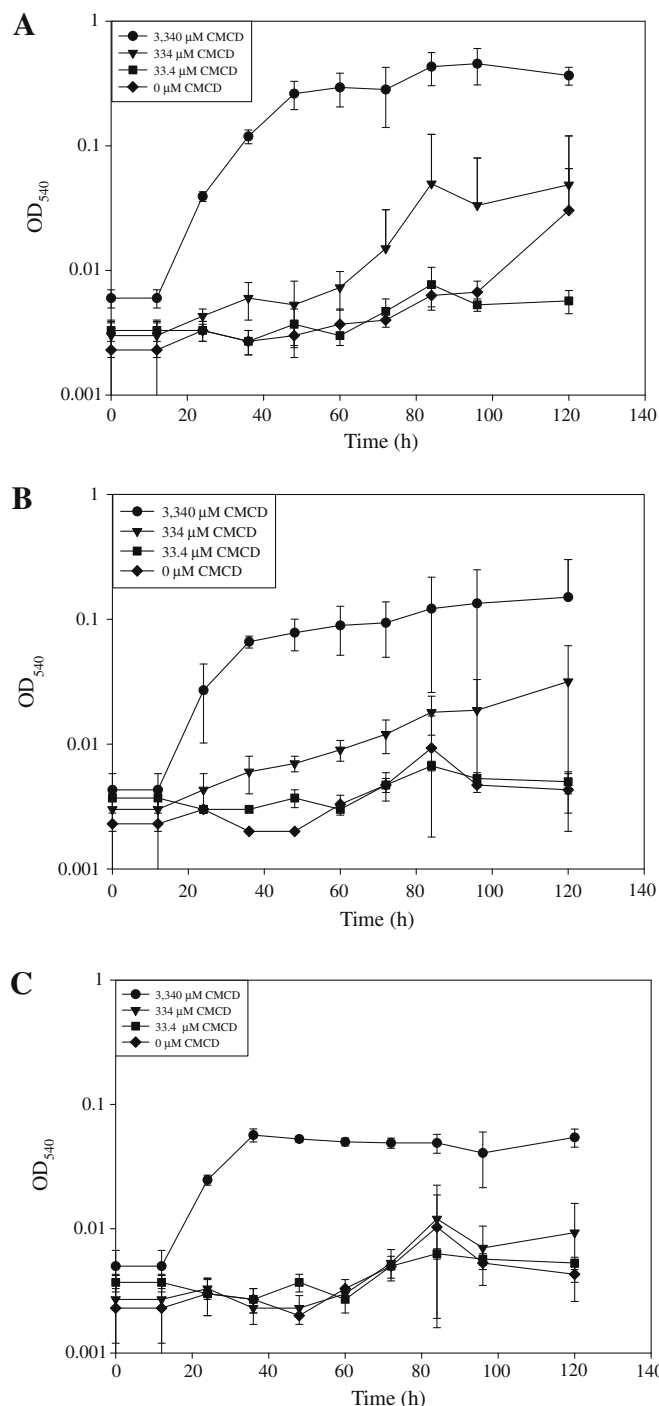

Fig. 2. Effect of carboxymethyl-β-cyclodextrin (CMCD) on growth of a naphthalene-degrading *Burkholderia* sp. in the presence of 33.4 μM cadmium (A), cobalt (B), and copper (C). Points represent the mean OD_{540} of triplicate flasks. Bars represent one standard deviation above and below each mean.

lue of the constant for CMCD is only 3.66 (Wang and Brusseau, 1995), demonstrating that CMCD has a lower affinity for cadmium than rhamnolipid.

3.4. Effect of CMCD and HPCD on growth during naphthalene biodegradation in the absence of metals

We examined the ability of CMCD to enhance naphthalene biodegradation in the absence of metals to determine whether some of the observed protective effect against metal toxicity resulted,

Table 2
Effect of CMCD on growth kinetics of *Burkholderia* sp. on naphthalene in the absence of toxic metals. Values of the same parameter followed by the same letter are not significantly different at $P = 0.05$ as measured by the Least Significant Difference (LSD) test.

	[CMCD] (μM)			
	0	33.4	334	3340
Lag phase (h)	65.3a	36a	12b	12b
Yield (OD$_{540}$)	0.17b	0.25ab	0.39a	0.20b
Log phase growth rate (OD$_{540}$/h)	0.006a	0.007a	0.003a	0.008a

at least in part, from the ability of CMCD to enhance the solubility of the naphthalene substrate. While the growth rate was unaffected, CMCD (334 and 3340 μM) reduced the lag phase by 53.3 h (Table 2). In addition, the cell yield of cultures amended with 334 μM CMCD was higher (OD$_{540}$ = 0.39) than in the other treatments. Taken together, these data suggested that the mechanism by which CMCD ameliorated metal toxicity during naphthalene biodegradation also involved CMCD enhancing naphthalene bioavailability.

To further investigate the importance of the ability of CMCD to enhance naphthalene bioavailability in the observed reduction in cadmium toxicity, we examined the ability of hydroxypropyl-β-cyclodextrin (HPCD) to reduce cadmium toxicity during naphthalene biodegradation. HPCD is a cyclodextrin derivative with hydroxypropyl substituents. Similar to CMCD, HPCD has been shown to enhance the solubilization of naphthalene (e.g., Badr et al., 2004), but HPCD lacks the metal-binding carboxymethyl substituents of CMCD. To our knowledge, no data has been published regarding solubility enhancements for naphthalene by CMCD; however, Wang and Brusseau (1995) found that a 10 g/L aqueous solution of CMCD enhanced the apparent solubility of anthracene by 9 times that of water. That level of enhancement is less than the 30-fold enhancement determined from the results by McCray (2000) for anthracene with a 10 g/L solution of HPCD.

Not surprisingly, HPCD did not reduce Cd^{2+} concentrations as measured by an ion-selective electrode (data not shown). In addition, HPCD failed to increase growth in the presence of cadmium (data not shown). Assuming that HPCD enhances the solubility and bioavailability of naphthalene at least as effectively as CMCD in the absence of metals, these data strongly suggest that the ability of CMCD to complex metals plays an important role in its ability to enhance growth during naphthalene biodegradation in the presence of toxic metals.

4. Conclusions

Our data demonstrate that CMCD can mitigate metal toxicity during naphthalene biodegradation. A 100-fold greater concentration of CMCD than each toxic metal was required to ameliorate toxicity. This performance compares favorably with a previous approach to mitigate cadmium toxicity during naphthalene biodegradation that used surfactant-modified clay (Malakul et al., 1998), but less favorably than an approach employing a biological surfactant (Sandrin et al., 2000). The mechanism of protection appears reliant upon the metal-complexing ability of CMCD. Cyclodextrins have shown promise in facilitating organic pollutant removal in the field (Blanford, 2001; McCray, 2000; Tick, 2003). Given this application and its promise, metal-binding modified cyclodextrins might be used to accelerate remediation efforts by concomitantly augmenting pollutant removal and biodegradation in co-contaminated environments. Further research is necessary to determine the practicality and efficacy of such an approach.

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