MECHANISM OF NONIONIC SURFACTANT INHIBITION OF PENTACHLOROPHENOL BIODEGRADATION

T. Cort and A. Bielefeldt

University of Colorado Boulder, Department of Civil, Environmental and Architectural Engineering, Campus Box 428, Boulder, CO 80309; Phone: (303) 492-8433; Fax (303) 492-7317. E-mail: cort@ucsu.colorado.edu

ABSTRACT

Several potential mechanisms of surfactant-induced inhibition of pentachlorophenol (PCP) biodegradation were tested using a pure bacterial culture of *Sphingomonas chlorophenolicum* sp. Strain RA2. Concentrations of the surfactant Tergitol NP-10 (TNP10) over 200 mg/L inhibit biodegradation of PCP at concentrations below 100 mg/L. At PCP concentrations above 200 mg/L, TNP10 reduced the substrate inhibition effect of PCP, resulting in faster PCP degradation rates at higher concentrations of TNP10. Measurements of surfactant confirm that TNP10 is not degraded by RA2. Calculations of a micelle partition coefficient (K_m) show that the differences in initial PCP degradation rates can be explained by accounting for the amount of PCP available to the cell in the aqueous solution. Also, TNP10 between 500 and 5000 mg/L did not slow glucose degradation or endogenous oxygen uptake rates, indicating a lack of general cellular toxicity from the TNP10. A model is discussed based on these results where PCP is sequestered into micelles at high TNP10 concentrations to become less available to the bacterial cell and resulting in observed inhibition as opposed to a mechanism of general cellular toxicity. Under substrate toxicity conditions, the same mechanism serves to increase the rate of PCP biodegradation by reducing aqueous PCP concentrations to less toxic levels.

Key words: *pentachlorophenol, bioremediation, surfactants, mechanism*

INTRODUCTION

The use of surfactants has the potential to increase the biodegradation rates of hydrophobic organic compounds in contaminated environments by increasing the total aqueous solubility of these compounds. However, inhibitory effects of surfactants on the biodegradation process have frequently been reported (Rouse et al., 1994). A variety of factors and mechanisms have been proposed to explain the inhibition process, including cellular toxicity from interaction of surfactant molecules with cell membranes (changing fluidity) or directly with membrane-bound proteins (Laha and Luthy, 1992), direct inhibition of enzymes involved in the catabolic pathway either by association with the enzyme or with the substrate (Gupte et al., 1995), decreased bioavailability due to sequestration of the substrate compound into surfactant micelles (Tiehm, 1994), or the accumulation of toxic intermediates due to incomplete metabolism incurred from substrate-surfactant interactions (Gupte et al., 1995). The future utility of surfactant-aided bioremediation efforts relies on the ability to accurately predict the effect of surfactants on a given bacterial culture with a known set of organic contaminants.

PCP is a widespread environmental contaminant of soils, surface waters, and groundwater (McAllister et al., 1996). PCP is a probable carcinogen and has been placed on the National Pollutants Priority List with a maximum drinking water concentration of 0.22 mg/L (US EPA, 1992). Over 678 sites in the United

States are contaminated with PCP, including 71 sites on the National Priorities List (USEPA, 1997).

MATERIALS AND METHODS

TNP10 and PCP Substrate Toxicity Tests

Batch tests were conducted in sterile flasks containing 50 ml of autoclaved minimal nutrient media (Widdel and Pfennig, 1977) at pH 6.8 that were capped with sterile glass wool and aluminum foil to prevent contamination. PCP (Sigma Chemical Co. 95%) was added from aqueous stock to produce ten flasks containing either 50, 100, 140, 220, or 300 mg/L. TNP10 (Sigma Chemical Co. 99%) was then added to form duplicate flasks of each surfactant concentration (0-3000 mg/L TNP10). Each experimental flask was inoculated with 5% by volume of liquid RA2 culture grown to late log phase on 100 mg/L PCP. Experimental sets included the following controls: 1) RA2 and 100 mg/L TNP10 only, 2) RA2 with no PCP or surfactant, and 3) 100 mg/L PCP and 100 mg/L TNP10 without RA2. Flasks were incubated in the dark at 10°C on a shaker table. Samples (3 ml) were taken every 5 to 24 hours, depending on growth rates. Biomass was measured spectrophotometrically at 600 nm and converted to dry mass measured as volatile suspended solids (VSS; *25*) by a standard curve. Samples were then centrifuged at 10,000 rpm for five minutes to settle cellular material, and the supernatant was measured for absorbance at 320 nm to indicate PCP concentration. Absorbance measurements were made on a Shimadzu UV160 UV-Visible Recording Spectrophotometer. Standard curves for PCP solutions containing TNP10 indicated that the surfactant concentration did not affect the absorbance of PCP.

Determination of Micelle Partitioning Coefficient

PCP (10 mg) was added into 100 ml of deionized water adjusted to pH 2 containing 0 to 1256 mg/L TNP10. Without surfactant, the solubility of PCP at pH 2 (25°C) is 2.98 mg/L (Arcand et al., 1995). Sterile batch bottles were sealed with aluminum crimp caps and allowed to shake in the dark at 25°C for one to two weeks. Liquid samples (10 ml) were then extracted from the crimped bottles by syringe and filtered through a 0.22 µm glass fiber filter to remove solid PCP. Five 10-ml volumes were extracted and filtered in this manner prior to the actual sample to wet the syringe, filter, and glassware so as to minimize sorption losses of surfactant and PCP. The final filtered sample was measured for PCP concentration via absorbance at 320 nm.

Surfactant Toxicity on Endogenous Decay and Glucose Metabolism

Cultures of RA2 (50 ml) were grown on 100 mg/L PCP to late log phase/early endogenous decay (PCP concentration <5 mg/L) and measured for biomass concentration. The decrease in dissolved oxygen (DO) in a closed, stirred flask due to endogenous decay was then measured by DO probe (YSI Model 5750 BOD probe and YSI Model 52 DO meter). After three hours, 0-5000 mg/L TNP10 was injected into the flask and DO was measured

for an additional three hours. A control with 100 mg/L PCP but no RA2 was tested to determine the effect of temperature fluctuation on DO measurements.

Cultures of RA2 (50 ml) were grown on 100 mg/L sterile filtered glucose (Sigma Chemical Co. 98%) to early log phase (~ one day) and measured for biomass concentration. Tests for oxygen uptake rate were performed in a similar manner as with endogenous decay rates as described above. During DO measurements, a replicate flask was run simultaneously in the same water bath. Both flasks were measured for DO uptake for 10 minutes (every 5 seconds). After 10 minutes, one of the flasks was injected with surfactant. The replicate flask was injected with an identical volume of distilled/deionized water at the same time. DO measurements were continued for an additional 10 minutes. Two additional controls containing 100 mg/L glucose and no RA2 were run for background changes in DO. One of the replicates was injected with 5000 mg/L TNP10 after 10 minutes to evaluate any changes in DO due to interaction of surfactant with glucose. Dissolved oxygen uptake rate was calculated in the same manner as endogenous decay tests, with the exception that data in the calculations were taken only 10 seconds after the system perterbation.

RESULTS

TNP10 and PCP Substrate Toxicity Tests

At 300 mg/L PCP and 10°C, PCP degradation by RA2 was completely inhibited under all surfactant conditions (0-1500 mg/L TNP10). Initial degradation rates at 220, 140, and 100 mg/L initial PCP under varying surfactant concentrations are shown in Table 1. The slow degradation rates at higher PCP concentrations without surfactant present indicate a severe substrate inhibition above 100 mg/L PCP. Below 100 mg/L PCP, substrate inhibition effects were not observed.

At high initial PCP concentrations, the addition of TNP10 enhanced PCP degradation rates. This is in contrast to previous results showing that at initial PCP concentrations of 50-100 mg/L, TNP10 addition inhibited PCP degradation (Cort and Bielefeldt, 2000). The initial rates of PCP degradation in Table 1 show that at 50 mg/L initial PCP, the degradation rate slows as TNP10 concentration increases,

Table 1. Initial PCP degradation rates (-d[PCP]/dt) at different TNP10 concentrations, in mg/L hr.

Initial $[PCP]_{total}$ (mg/L)	[TNP10] (mg/L)				
	1500	1000	500	100	0
300	0.00	-0.01	-0.01	0.01	-0.01
220	0.07	0.01	0.00	0.00	0.02
140	0.19	0.14	0.08	0.07	0.11
100	0.23	0.26	0.36	0.27	0.33
50	0.08	0.17	0.23	0.26	0.30

whereas at 220 mg/L initial PCP concentration, its degradation rate is highest with 1500 mg/L TNP10 addition. The overall rates at higher initial PCP concentrations without TNP10 are slower, indicating that TNP10 is serving to mitigate the substrate toxicity of PCP rather than enhance the PCP degradation rate over controls.

Determination of Micelle Partitioning Coefficient

To estimate the concentration of PCP partitioned into the micellar and free aqueous phase, the solubility of PCP in the presence of TNP10 was determined at pH = 2 to ensure that all of the PCP was un-ionized. PCP concentration increased linearly as surfactant was added in the range of 0-1256 mg/L TNP10, yielding a slope (Molar Solubilization Ratio, MSR) of 0.0017 molar PCP/molar TNP10. The MSR was used to calculate the micelle-phase/aqueous-phase partition coefficient, $K_{m,}$ based on the mole fractions of PCP in either phase (Edwards et al., 1991). The calculated K_m at pH = 2 is 1886. To convert to K_m at pH 7, the relationship of K_m to the octanol-water partition coefficient, K_{ow}, was utilized (Edwards et al., 1991). Edwards et al. (1991) have shown that the ratio of log K_m to log K_{ow} is approximately 1 for PAH compounds in TNP10 (Edwards et al., 1991). Assuming this ratio is true for PCP, since K_{ow} can account for the decreased hydrophobicity upon PCP acid dissociation, the K_{ow} values for PCP at a pH of 2 (log K_{ow} = 5) and 7 (log K_{ow} for sum of ionized and unionized PCP = 3) (Christodoulatos and Mohiuddin, 1996) were used to calculate an approximate K_m of 19 at pH = 7. This K_m value was used to estimate the free aqueous PCP concentrations from the measured total PCP concentration.

The initial rate of PCP degradation corrected for biomass was calculated for each degradation curve containing 0, 500, and 1500 mg/L TNP10 as a function of the aqueous initial PCP concentration at 10°C. Initial degradation rates for cultures containing 500 and 1500 mg/L TNP10 were also calculated assuming a micelle

Table 2. Initial PCP degradation rates corrected for biomass ($-(d[PCP]/dt)_o(1/X)$) at different TNP10 concentrations (hr^{-1}). Data shown with and without correction for micelle partitioning coefficient (K_m).

Initial $[PCP]_{total}$ (mg/L)	[TNP10] (mg/L)				
		Not Corrected for K_m		Corrected for K_m=19	
	0	500	1000	500	1000
50	0.30	0.23	0.08	0.33	0.13
100	0.33	0.36	0.23	0.41	0.32
140	0.11	0.08	0.19	0.10	0.30
220	0.02	0.00	0.07	0.03	0.12
300	-0.01	-0.01	0.00	0.00	0.00

partition coefficient of 19. Results are shown in Table 2. The observed rates of initial PCP biodegradation were much closer to the surfactant-free control after accounting for PCP sequestered into the micelle. This is particularly true at lower PCP concentrations where initial biodegradation rates were higher.

Surfactant Toxicity on Endogenous Decay and Glucose Metabolism

Measurements of dissolved oxygen (DO) uptake rate were conducted on RA2 cells during endogenous decay and glucose degradation. Endogenous decay measures the cellular respiration activity of the bacteria with no PCP present in order to isolate the effect of TNP10. Glucose metabolism tests utilized a substrate that is expected to partition very little into the surfactant micelle, thereby eliminating bioavailability as a possible inhibition mechanism. Table 3 shows the DO uptake rate before and after the addition of varying amounts of TNP10. It is apparent, based on the percent changes in DO uptake, that TNP10 addition does not decrease the cell activity during endogenous decay or during glucose metabolism. The results suggest that TNP10 is not inherently toxic to RA2 cells. On the contrary, it appears that addition of surfactant increases the rate of endogenous decay. The increase in DO uptake is not due to surfactant degradation since

Table 3. Effect of TNP10 addition on endogenous decay and glucose metabolism, measured by dissolved oxygen uptake rate.

Test Type	[TNP10] (mg/L)	Initial Rate[a] (µg/L min)	Final Rate[b] (µg/L min)	% Change
End. Decay	0	2.3	1.7	-26
End. Decay	50	4.0	4.6	15
End. Decay	100	3.0	2.6	-15
End. Decay	1000	2.1	3.6	70
End. Decay	2000	1.5	4.9	223
End. Decay	3000	3.6	6.2	73
End. Decay	5000	1.5	4.8	230
Glucose Met.	0	2.4	2.4	0
Glucose Met.	100	7.1	5.4	-24
Glucose Met.	500	6.8	7.1	5
Glucose Met.	1000	5.4	4.4	-19
Glucose Met.	5000	2.2	5.7	155

[a] Average DO uptake rate before injection of TNP10.
[b] DO uptake rate after injection of TNP10.

batch tests found no decrease in surfactant concentration and no increase in biomass concentration when RA2 and TNP10 were incubated together.

DISCUSSION

TNP10 does not appear to inhibit PCP biodegradation by a mechanism of general cellular toxicity. First, the dissolved oxygen uptake rates of RA2, after addition of TNP10, show that less than 5000 mg/L surfactant does not inhibit the cell metabolism. Since PCP is not present, this uptake rate reflects the general cell metabolism of RA2 rather than any effects unique to interaction between the hydrophobic substrate and the surfactant molecules. However, an increase in endogenous decay rates was observed after addition of high TNP10 concentrations. This suggests that cellular activity actually increases with surfactant addition, arguing strongly against a general mechanism of cellular toxicity. Similarly, the addition of TNP10 did not inhibit DO uptake rates during growth on glucose. It is known that glucose partitions very little into surfactant micelles due to its polar nature. This suggests that the inhibition effect could be explained by the decrease in available, aqueous-phase PCP in the presence of higher concentrations of surfactant micelles. Second, at high concentrations of PCP, TNP10 increases the biodegradation rate of PCP at TNP10 concentrations as high as 1500 mg/L. This result cannot be explained by a mechanism of inhibition inherent to the surfactant interaction with the cell.

The observation of enhanced PCP degradation rates in the presence of high surfactant concentrations also argues against an inhibition mechanism involving catabolic enzymes. It is difficult to hypothesize a mechanism of surfactant inhibition on catabolic enzymes that can be reversed in the presence of high PCP concentrations. The mitigation of this PCP substrate toxicity by TNP10 also suggests that TNP10 is not acting on just the catabolic enzymes. Finally, initial results in this lab indicate that a classic, competitive enzyme-inhibition model, based on Michaelis-Menten kinetics, is inadequate to explain the observed inhibition of PCP degradation by TNP10 between 0-100 mg/L initial PCP (Cort and Bielefeldt, 2000).

The bacterial system used in these tests precludes the influence of metabolic intermediates in the surfactant effects. The rate-limiting step in PCP degradation has been shown to be the first enzymatic step (monooxygenation of PCP to tetra-chlorohydroquinone) (McAllister et al., 1996) so that metabolites of PCP degradation do not accumulate in whole cell systems. Moreover, the intermediate aromatic metabolites tetra-, di-, and mono-chlorohydroquinones absorb in the visible light region. No color change was seen in cell cultures during TNP10 inhibition studies to indicate the accumulation of these intermediates.

It appears that micelle sequestration is a likely predominant mechanism for TNP10 inhibition of PCP biodegradation in aqueous systems. This model predicts that similar rates of PCP degradation will be observed for similar free aqueous PCP concentrations. By accounting for the PCP sequestered in the micelle, the initial degradation rates for solutions containing

0-1500 mg/L TNP10 begin to overlap (Table 2), which is consistent with the sequestration model. Calculations utilizing the micelle partitioning coefficient also indicate that at high TNP10 concentrations, enough PCP partitions into the micelle to decrease the aqueous PCP concentrations to non-toxic levels. This model also accounts for the observed experimental results. Below the threshold of PCP toxicity (~150 mg/L at 10°C), added surfactant can inhibit PCP degradation rates because PCP partitioned into the micelle is unavailable to the RA2 cell. The rate then becomes dependent on partitioning rates of PCP between micelle, aqueous, and cell phases. Above 150 mg/L PCP, the surfactant enhances PCP degradation rates by reducing aqueous, 'available' PCP concentrations below the threshold of toxicity.

Surfactant-enhanced bioremediation applications depend on a faster overall biodegradation rate of the target compound. Due to the decreased availability of PCP sequestered into the micelle, such an application will only be beneficial if the surfactant increases the rate of PCP desorption from the soil or dissolution from nonaqueous-phase liquids. Also, the transfer rate of PCP between soil, water, and micelle phases must be greater than the rate of soil-to-water transfer in the absence of surfactants. In this way, surfactant micelles could increase the overall transfer rate of PCP from soil to the aqueous phase. Further work is needed to measure the mass transfer rates between different phases and to determine if this micelle-sequestering mechanism is common to other bacteria and organic contaminants.

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REFERENCES

Arcand, Y., J. Hawari, and S.R. Guiot, 1995, Solubility of pentachlorophenol in aqueous solutions: the pH effect, Wat. Res., vol. 29, pp. 131-136.

Christodoulatos, C., and M. Mohiuddin, 1996, Generalized models for prediction of pentachlorophenol adsorption by natural soils, Water Env. Res., vol. 68, pp. 370-378.

Cort, T., and A.R. Bielefeldt, 2000, Effects of surfactants and temperature on PCP biodegradation, J. Environ. Eng., vol. 126, pp. 635-643.

Edwards, D.A., R.G. Luthy, and Z. Liu, 1991, Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions, Environ. Sci. Technol., vol. 25, pp. 127-133.

Gupte, A., R. Nagarajan, and A. Kilara, 1995, Enzymatic oxidation of cholesterol in reverse micelles, Ind. Eng. Chem. Res., vol. 34, pp. 2910-2922.

Laha, S., and R.G. Luthy, 1992, Effects of nonionic surfactants on the solubilization and mineralization of phenanthrene in soil-water systems, Biotechnol. Bioeng., vol. 40, pp. 1367-1380.

McAllister, K.A., H. Lee, and J.T. Trevors, 1996, Microbial degradation of pentachlorophenol, Biodegradation, vol. 7, pp. 1-40.

Rouse, J.D., D.A. Sabatini, J.M. Suflita, and J.H. Harwell, 1994, Influence of surfactants on microbial degradation of organic compounds, Crit. Review Env. Sci. Technol., vol. 24, pp. 325-370.

Tiehm, A., 1994, Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants, Appl. Environ. Microbiol., vol. 60, pp. 60-67.

Widdel, F., and N. Pfennig, 1977, A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, *Desulfotomaculum (*emend.) *aetoxidans,* Arch. Microbiol., vol. 112, pp. 119-122.

US EPA, 1992, Contaminants and remedial options at wood preserving sites, EPA/600/R-92/182 US EPA, Washington D.C.

US EPA, 1997, Treatment technology performance and cost data for remediation of wood preserving sites, EPA/625/R-97/009 US EPA, Washington D.C.