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Copper Toxicity towards a Pentachlorophenol-Degrading *Flavobacterium* sp.

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Increasing public concern over the contamination of the environment with industrial wastes has elicited considerable research into methods of removing them from soil and water (Cork and Krueger 1991). Bioremediation is a microbial technique that has been proposed as an alternative to more conventional waste management processes such as charcoal trapping, incineration and landfill disposal (Topp and Hanson 1990). However, contaminated sites normally contain a complex mixture of toxicants and this often limits the field application of this technology (Haggblom 1992), usually as a result of inhibitory effects elicited by the co-occuring toxicants. Consequently, detailed information is required on biodegradation processes in the presence of multiple toxicants in order to accurately assess the feasibility of using bioremediation techniques. Unfortunately, these data are generally unavailable. The wood-preserving industry is an ideal model for examining these processes since only a limited number of chemicals are used.

In North America, creosote, pentachlorophenol (PCP), and aqueous formulations of arsenic, copper, and either chromium or ammonia are the chemicals most commonly used for wood preservation (Konasewich and Henning 1988 a,b,c). Combinations of these toxicants are commonly found in the soil and ground water near wood-preserving facilities (Lamar et al. 1990). PCP is of particular concern as it is a priority pollutant that exhibits broad spectrum toxicity and is a potential carcinogen (reviewed in Seiler 1991). A *Flavobacterium* sp. (ATCC 53874) capable of utilizing PCP as a sole carbon source has been isolated (Saber and Crawford 1985) and substantial progress has been made towards the field application of this bacterium for the *in situ* biodegradation of PCP (Seech et al. 1991). However, complex questions regarding the effect of co-occuring toxicants on the growth and metabolic activity of *Flavobacterium* remain to be addressed. Toxic metals are common contaminants of natural waters and soils and may adversely affect biodegradation processes (Said and Lewis 1991). Chromium, copper and arsenic, which are the active ingredients of chromated-copper-arsenate (CCA) wood preservatives, are often found in association with PCP contamination. CCA formulations are known to inhibit PCP degradation by *Flavobacterium* (Topp and Hanson 1990). However, the effects of the constituent metals found in CCA on PCP degradation have not been evaluated. The purpose of the present study was to examine the effects of copper (as Cu^{+2}) on the growth and metabolic activity of a PCP-degrading *Flavobacterium* sp. Such information is essential if accurate predictions are to be made regarding the biodegradation of PCP in metal-contaminated environments.

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MATERIALS AND METHODS

The bacterial culture used was *Flavobacterium* sp. ATCC 53,874 (Am. Type Culture Collection, Rockville, MD, USA). Stock cultures were maintained at 30°C in a liquid mineral salts (MS) growth medium supplemented with 4.0 g L^{-1} of sodium glutamate (Saber and Crawford 1985). Cultures used as an inoculant were incubated in 250 mL Erlenmeyer flasks for 48 hr on a rotary shaker at 125 rpm.

Copper sulfate ($CuSO_4 \cdot 7\ H_20$; >99% purity; Aldrich Chemical Co., Milwaukee, WI., USA) was used as the toxicant in all bioassays. Stock solutions were prepared using glass distilled water and were filter sterilized prior to use (0.45 μm membrane filter). All copper concentrations are given as μg mL^{-1} of Cu^{+2}. Pentachlorophenol (PCP; >99% purity; Sigma Chemical Co., St. Louis, MO, USA) was dissolved in acetone and added to test systems from a filter sterilized 10,000 μg mL^{-1} stock solution. Concentrations of 10, 25, 50, 75 and 100 μg mL^{-1} were employed in studies examining the effects of Cu^{+2} on PCP degradation by *Flavobacterium*. The background acetone level was standardized at 1.0 % vol vol^{-1}.

Toxic effects of Cu^{+2} on the growth of *Flavobacterium* were determined using test tube bioassays. *Flavobacterium* cells were inoculated to a final concentration of $1x10^6$ cells mL^{-1} into 18 x 150 mm glass test tubes containing MS medium. $CuSO_4$ was added aseptically to a Cu^{+2} concentration ranging from 0.1 to 100 μg mL^{-1}. Total assay volume was 10 mL. Test tubes were incubated in darkness at 30 ± 1°C and bacterial growth monitored spectrophotometrically at 660 nm. Optical density readings were taken at 24 hr intervals and corrected for deviations from Beer's law (Koch 1981). Growth in Cu^{+2}-treated systems was compared to that evidenced by the controls (no Cu^{+2} present) and percent inhibition values calculated. All treatments were replicated five times.

The effects of Cu^{+2} on PCP degradation by *Flavobacterium* were assessed using batch cultures. *Flavobacterium* cells were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of MS medium (final level of $1x10^6$ cells mL^{-1}). Cu^{+2} solutions were added at levels corresponding to the 96 hr EC_{25}, EC_{50} and EC_{75} values, as calculated towards bacterial growth. Test systems were incubated at 30°C at 125 rpm on a Lab-Line environmental shaker (Model 3528, Lab-Line Instruments, Inc., Melrose Park, ILL, USA). All degradation experiments were replicated five times. PCP was extracted from the MS medium using a C_{18} column (Sep-Pak Vac; Millipore Corp., Mississauga, Ont., Canada) according to the procedure outlined in Wall and Stratton (1991). PCP concentrations were quantified by high performance liquid chromatography, as outlined elsewhere (Wall and Stratton 1991). All solvents used were HPLC grade (Caledon Laboratories, Georgetown, Ont., Canada).

Growth curves were prepared by plotting corrected optical density values versus time. Dose-response curves were constructed every 24 hr by plotting the percent inhibition of growth versus the $\log_{10}$ of Cu^{+2} concentration. The resulting curve was fitted with a second order polynomial equation using the Cricket Graph graphics package (Cricket Software, Malvern, PA, USA). These equations were used to mathematically determine the EC_{25}, EC_{50}, and EC_{75} values for Cu^{+2}, based upon the inhibition of growth yield (optical density). Significant differences at $P \leq 0.05$ were determined using either an analysis of variance procedure followed by a Tukey's Studentized range test, or a Student's t-test, where applicable (SAS Statistics Software, SAS Institute, Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

The present study was designed to investigate the effects of copper (as Cu^{+2}) on the growth and PCP degrading activity of *Flavobacterium* sp. ATCC 53874. CuO is the chemical used to supply Cu^{+2} in the commercial preparation of CCA wood preservatives (Konasewich and Henning, 1988b), but its lack of solubility in our test system precluded its use. $CuSO_4$, which was highly soluble in the test systems and which supplies the proper chemical species (Cu^{+2}), was substituted for CuO. Fifteen Cu^{+2} concentrations were tested towards growth of *Flavobacterium*, yielding 15 separate bacterial growth curves where growth was monitored as an increase in optical density over time. Representative growth curves are presented in Fig. 1. Optical density values were also converted to $\log_e$ and replotted to further evaluate growth curve patterns (data not shown).

Copper completely inhibited bacterial growth at concentrations $\geq$ 90 $\mu g\ mL^{-1}$. Cu^{+2} levels below this did not have a pronounced effect on the lag phase of growth but did noticeably inhibit the growth rate during the early logarithmic phase (Fig. 1). These effects were more apparent at the higher Cu^{+2} concentrations. The time required for the culture to enter into the stationary phase of growth did not appear to be markedly affected by Cu^{+2} (Fig. 1). However, total cell yields (maximum amount of growth attained) in Cu^{+2}-treated systems were significantly lower than in the controls, and never recovered to control levels during the 21 day incubation period (Fig. 1). Extrapolation of the curves past this 21 day period suggested that cell yield was permanently and irreversibly reduced in Cu^{+2}-treated systems. The degree of reduction was dose-dependent.

Dose-response curves were prepared at 24 hr intervals for the aqueous copper formulation tested (curves not shown). Each curve was then fitted with a second-order polynomial equation (all $R^2>0.85$). These equations were used to calculate daily EC_{25}, EC_{50}, and EC_{75} values, which are the effective concentrations of Cu^{+2} which produced the degree of growth inhibition noted. The dose-response curve prepared after 96 hr of incubation was used as a reference for comparison purposes ($y = 6.80+3.93x+18.00x^2$; y = % inhibition; x = $\log_{10}$ Cu^{+2} concentration), because this is the time period required for *Flavobacterium* sp. ATCC 53874 to completely degrade PCP at concentrations up to, and including, 100 $\mu g\ mL^{-1}$. As well, a 96 hr incubation time is routinely cited in toxicity studies in aqueous bioassay systems (Greenberg et al. 1981). The Cu^{+2} concentrations required to produce a 96 hr EC_{25}, EC_{50}, and EC_{75} response were 8.0, 27.8 and 69.2 $\mu g\ mL^{-1}$, respectively.

In bacterial growth bioassays where cell yield (as quantified by optical density) is used to determine the toxicity response, the calculated toxicity parameters tend to vary somewhat from day to day. Although the 96 hr EC_{25}, EC_{50}, and EC_{75} values were used for reference purposes, these calculated toxicity parameters fluctuated substantially over time (data not shown). The same general trend in response was noted for all three toxicity parameters (EC_{25}, EC_{50}, and EC_{75}), in that the Cu^{+2} formulations tended to increase in toxicity (as evidenced by decreasing EC_{xx} values) until about 10 days post inoculation. The toxicity of the Cu^{+2} then gradually decreased over the remaining 11 day incubation period.

Copper was also tested towards PCP degradation by *Flavobacterium* sp. ATCC 53874. PCP degradation was examined using 5 concentrations of PCP in both the presence and absence of the three concentrations of Cu^{+2} that corresponded to the 96 hr EC_{25}, EC_{50}, and EC_{75} toxicity parameters. In total, 20 PCP degradation curves were obtained. Representative degradation curves are presented in Fig. 2. In control systems, PCP concentrations of 100 $\mu g\ mL^{-1}$ were completely degraded to

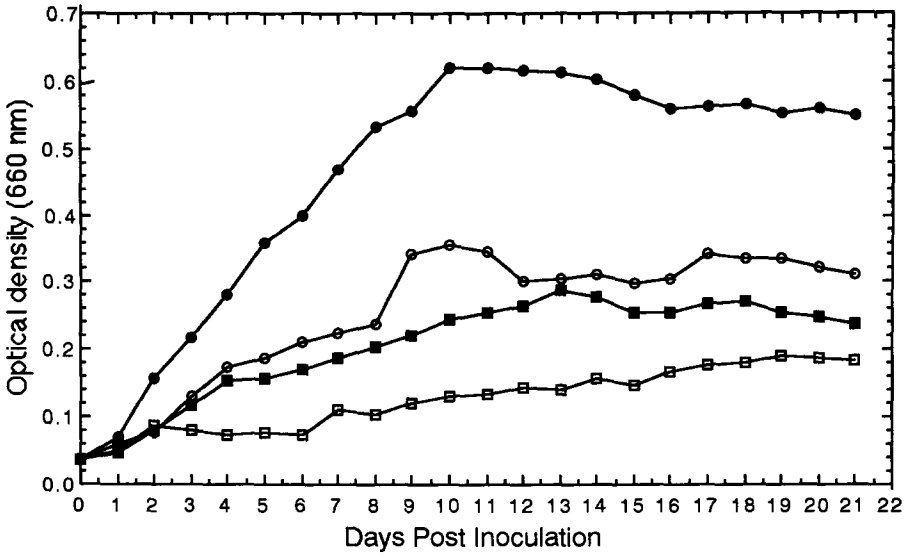

Fig. 1. Effects of Cu^{+2} on the growth of *Flavobacterium* sp.
Symbols: (●) control; (○) 8.0 μg Cu^{+2} mL^{-1}; (■) 27.8 μg mL^{-1}; (□) 69.2 μg mL^{-1}.

non-detectable levels within 56 hr (Fig 2a). Correspondingly less time was required for complete degradation of lower concentrations of PCP in the control systems. Although the same generalized shape was evidenced by each of the control degradation curves, a PCP concentration-dependent increase in the lag time prior to measurable PCP degradation was apparent. At a PCP concentration of 100 μg mL^{-1} (Fig 2a), 18 hr were required before measurable PCP degradation was observed, while the lag period was 10 to 12 hr at a PCP concentration of 75 (Fig. 2b) or 50 μg mL^{-1} (Fig. 2a). When the PCP concentration was reduced to 25 (Fig. 2b) or 10 μg mL^{-1} (data not shown), the apparent lag time decreased to 4 to 6 hr.

The representative curves in Fig. 2 (data for 100, 75, 50, and 25 μg PCP mL^{-1}) illustrate the pronounced effect Cu^{+2} had on PCP degradation by *Flavobacterium*. Although Cu^{+2} did not dramatically alter the generalized shape of the PCP degradation curves relative to the controls, or the rate of PCP degradation by *Flavobacterium*, it did elicit an increase in the lag time observed prior to measurable PCP degradation. A Cu^{+2} concentration of 8.0 μg mL^{-1} (EC_{25}) caused a significant increase in the lag time for PCP degradation only when tested in the presence of PCP concentrations that exceeded 50 μg mL^{-1}. However, at a Cu^{+2} concentration of 27.8 μg mL^{-1} (EC_{50}), a significant increase in the apparent lag time was observed at all PCP levels tested. Degradation was completely inhibited at PCP concentrations $\geq$ 50 μg mL^{-1} when *Flavobacterium* cells were exposed to a Cu^{+2} level of 69.2 μg mL^{-1} (EC_{75}). These Cu^{+2}-induced increases in the lag period of PCP degradation (Fig. 2a,b) also resulted in a significant increase in the overall time required to completely degrade PCP to non-detectable levels (Fig. 3). As the PCP concentration decreased, the percent increase in the time required for PCP degradation increased (Fig. 3). A similar response pattern was noted for both Cu^{+2} concentrations of 8.0 (EC_{25}) and 27.8 μg mL^{-1} (EC_{50}). The trend was less pronounced at a Cu^{+2} concentration of 69.2 μg mL^{-1} (EC_{75}), since PCP degradation was completely inhibited in most of the test systems at this Cu^{+2} concentration.

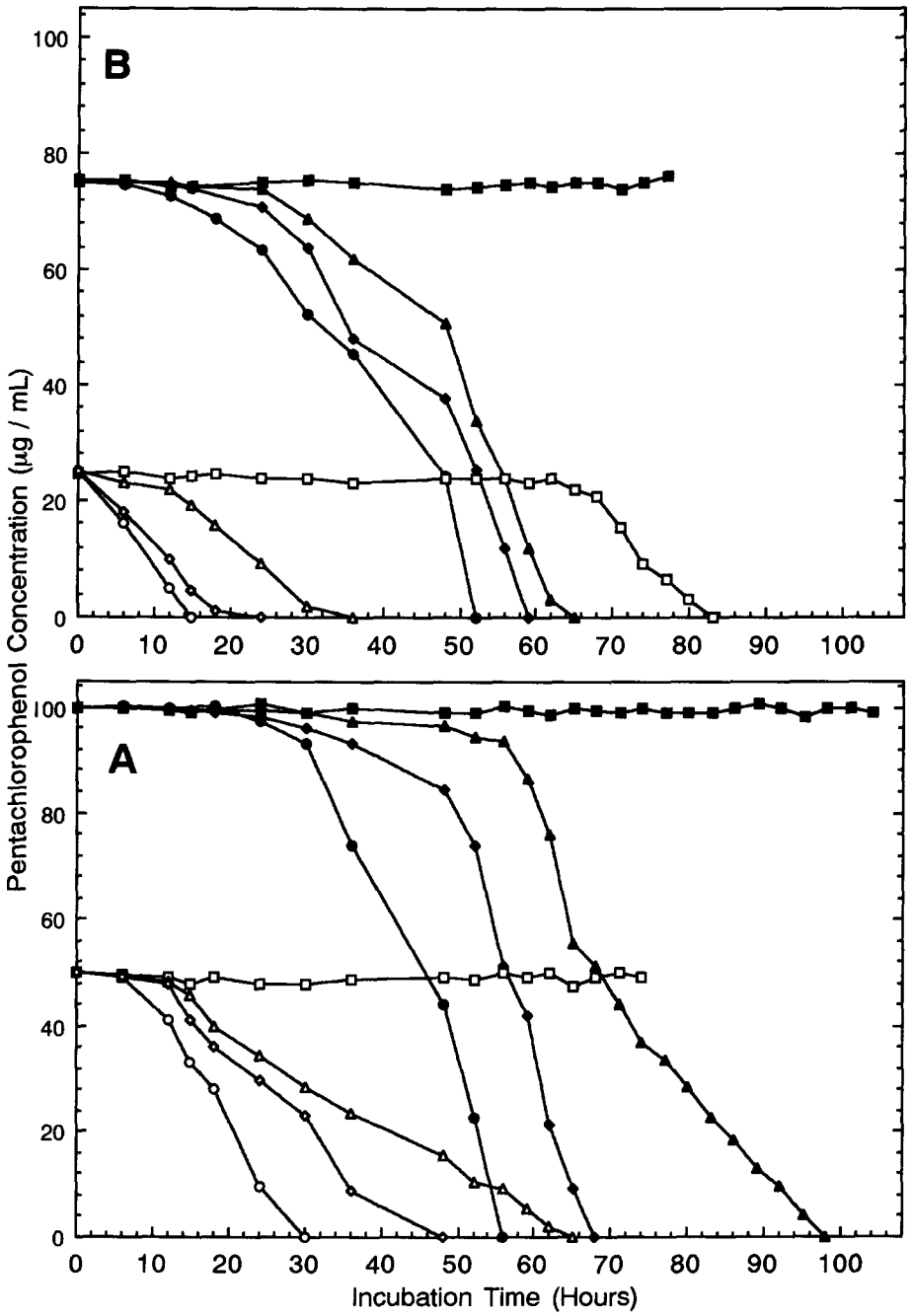

Fig. 2. Effects of Cu^{+2} on the degradation of PCP by *Flavobacterium*. The Cu^{+2} concentrations used were (●) control; (♦) 8.0 µg Cu^{+2} mL^{-1}; (▲) 27.8 µg mL^{-1}; (■) 69.2 µg mL^{-1}. **Fig. 2A.** Closed symbols: Degradation of 100 µg of PCP mL^{-1}; Open symbols: 50 µg of PCP mL^{-1}. **Fig. 2B.** Closed symbols: Degradation of 75 µg of PCP mL^{-1}; Open symbols: 25 µg of PCP mL^{-1}. The data for 10 µg of PCP mL^{-1} are not shown.

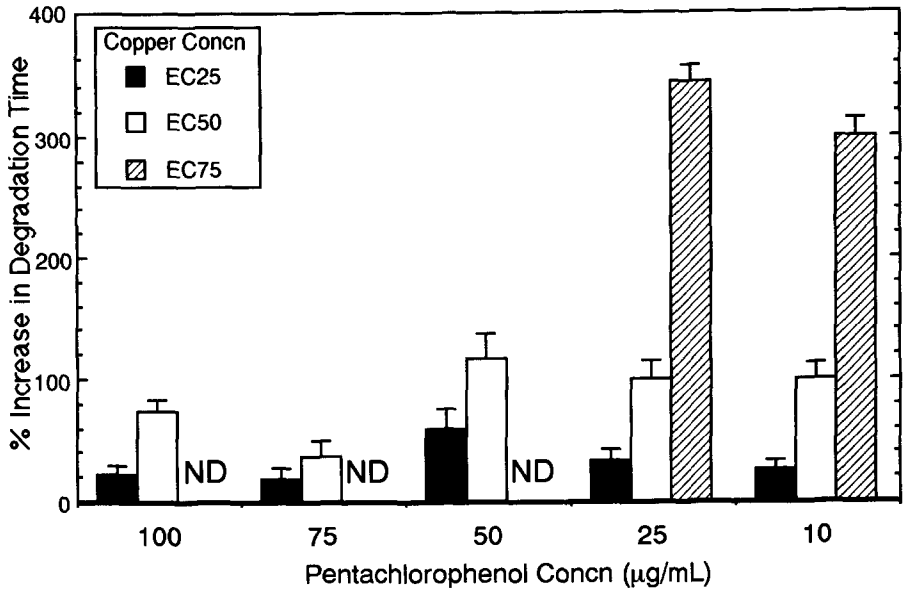

Fig. 3. Effect of Cu^{+2} on the time required for the complete degradation of PCP. Symbols: see graph. ND-no degradation noted. Vertical lines are standard deviation bars.

In the present study, Cu^{+2} concentrations exceeding 8.0 µg mL^{-1} significantly inhibited the growth and PCP-degrading activity of *Flavobacterium* sp. ATCC 53874. This is well below the Cu^{+2} concentration within a CCA working solution. The CCA concentrate sold commercially contains around 9 to 10% wt vol^{-1} of CuO (Konasewich and Henning 1988b). This concentrate is first diluted with water to a 1.5 to 4.0% v/v working solution before being applied to wood in a pressure cylinder (Konasewich and Henning 1988b). This would yield Cu^{+2} levels of between 1000 and 1200 µg mL^{-1}. There are few other data on the effects of Cu^{+2}-containing formulations towards PCP-degrading bacteria with which to compare the results outlined above. Topp and Hanson (1990) reported that CCA mixtures of 2/2/10 (mg L^{-1} Cu^{+2}, Cr^{+6}, and As^{+5}) significantly inhibited *Flavobacterium* growth, and completely blocked PCP degradation. Wall and Stratton (unpublished data) observed CCA to be even more toxic to *Flavobacterium*. A laboratory-prepared CCA formulation containing Cu^{+2}, Cr^{+6}, and As^{+5} at concentrations of 0.514, 0.313, and 0.485 µg mL^{-1}, respectively, caused a 50% reduction in growth of *Flavobacterium* and significantly decreased PCP degradation. A commercial CCA formulation evidenced an even higher toxicity. Other toxicity data are unavailable.

Although Cu^{+2} had no pronounced effect on the lag phase of growth, it did significantly inhibit the growth rate during the early logarithmic phase of growth (Fig. 1). The arrival of the stationary phase of growth was not significantly affected by Cu^{+2}, but a permanent reduction in cell yield was noted. Reasons for this response are presently unknown. There was also some change in Cu^{+2} toxicity over time, with the Cu^{+2} formulation generally attaining its maximum toxicity after 10 days of incubation. This increase in toxicity may be related to Cu^{+2} transport into the *Flavobacterium* cells. Metal transport into bacteria requires a fixed period of time and usually occurs in several stages. Toxicity would be expected to increase

until metal concentrations within the cell stabilized, following which cell division would effectively dilute metal exposure over time and reduce its toxicity. Cells could also recover from the initial acute Cu^{+2} exposure by chemically altering the metal via oxidation/reduction reactions and other microbially-mediated reactions (summarized in Flemming and Trevors 1989). *Flavobacterium* cells also produce significant amounts of extracellular polysaccharides (Saber and Crawford 1985), which may also bind metals and reduce their bioavailability and apparent toxicity (Flemming and Trevors 1989).

The growth medium itself can significantly influence metal toxicity, since all growth media contain a variety of organic and inorganic components which can complex with metals to reduce their bioavailability. Copper is particularly prone to such complexation. The medium used in the present study has a relatively high phosphate content and contains significant amounts of Mg^{+2}, both of which can buffer the biocidal effects of Cu^{+2} (Zevenhuizen et al. 1979). The medium used here was chosen because it is commonly employed in biodegradation studies with PCP-degrading *Flavobacterium* (Saber and Crawford 1985; Topp and Hanson 1990).

The effects of Cu^{+2} on PCP degradation by *Flavobacterium* were also investigated. Over the range of concentrations examined, increasing PCP levels did not alter the generalized degradation curve or the rate of PCP degradation. However, a direct relationship between increasing PCP concentration and the time interval between inoculation and measurable PCP degradation was observed (Fig. 2). Previously noted with other PCP-degrading bacteria (Radehaus and Schmidt 1992), this relationship between increasing organic toxicant concentration and lag time has been reported in other bacterial species capable of biodegrading organic compounds (e.g. Wiggins and Alexander 1988). The lag time prior to observable degradation is believed to be a function of both the time required for acclimation and accumulation of sufficient biomass to obtain measurable rates of degradation (Topp et al. 1988). A number of mechanisms, which include the time required for enzyme induction, genetic exchange or mutation, or the preferential use of other organic substrates, may also account for the acclimation period. It is possible that the *Flavobacterium* cells, which were uninduced to PCP prior to inoculation, required an additional period of acclimation when exposed to Cu^{+2}. This response would be expected with increasing concentrations of co-contaminants (Wiggins et al. 1987). Cu^{+2} also caused an increase in the amount of time required to completely degrade PCP (Fig. 3). In addition to possible effects on the acclimation period, the inhibitory effects of Cu^{+2} on growth as described above could have resulted in a decreased biomass, which would also require a longer time to recover to levels able to degrade PCP.

The data presented here indicate that copper co-contamination could significantly inhibit pentachlorophenol degradation by *Flavobacterium* and possibly other bacterial strains. This raises concerns regarding the use of bacteria to remove PCP residues from copper contaminated soils, and illustrates the need for further research concerning the biodegradation of toxicants in complex mixtures.

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