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6. AUTHOR(S)
Barbara R. SharaK Genthner, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of West Florida
Center for Environmental Diagnostics & Bioremediation
11000 University Parkway
Pensacola, FL 32514

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13. ABSTRACT (Maximum 200 words)

Heavy metal pollution often co-occurs with aromatic pollution. Therefore, we investigated the effect of added Cd(II), Cr(VI), Cu(II) and Hg(II) on biodegradation of 3-chlorobenzoate (3CB), 2-chlorophenol (2CP), phenol, and benzoate. [Cr(VI) was abiotically reduced to Cr(III) in our anaerobic medium, and Cu(II) partially reduced to Cu(I).] Complete anaerobic biodegradation of 3CB and 2CP is performed by an interdependent cooperation of anaerobic bacterial strains via 3CB → benzoate → acetate/H_2/CO_2 → methane; and 2CP → phenol → benzoate → acetate/H_2/CO_2 → methane. Target compounds were not degraded at >5-100ppm, while ≤0.1ppm Cd(II), Cu(II), or Cr(III) had no effect. 0.1ppm Hg(II) inhibited 2CP and phenol, but not 3CB or benzoate, biodegradation. Cd(II) and Cr(III) inhibited 3CB dechlorination most strongly. Cu(II) and Hg(II), respectively, inhibited benzoate and phenol biodegradation most strongly. Utilization of acetate was most sensitive to Cu(II) and Hg(II). Methane production was most sensitive to Hg(II). No correlation existed between the initial Eh created by adding metal salts to anaerobic medium and observed inhibition of target compound biodegradation. 2CP and 3CB biodegradation became resistant to Hg(II) by an inducible, transferable, biological activity which is under further investigation.

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FINAL REPORT

Grant #: N00014-93-1-1222

PRINCIPAL INVESTIGATOR: Dr. Barbara R. Sharak Genthner

INSTITUTION: Center for Environmental Diagnostics & Bioremediation - University of West Florida, Pensacola, FL

GRANT TITLE: Effect of Metals on the Biotransformation of Chlorinated and Non-chlorinated Aromatic Compounds by Anaerobic Bacterial Consortia

AWARD PERIOD: 1 October 1993 - 31 December 1996

OBJECTIVE: To investigate the effect of heavy metal pollutants [Cu(II), Cd(II), Cr(VI), and Hg(II)] on anaerobic biodegradation of selected chlorinated [2-chlorophenol (2CP) and 3-chlorobenzoate (3CB)] and non-chlorinated (phenol and benzoate)aromatic compounds.

APPROACH: Complete biodegradation of 3CB proceeds by dechlorination to benzoate; biodegradation of benzoate to acetate, H_2, and CO_2; and utilization of acetate, H_2, and CO_2 for methanogenesis. Due to unfavorable thermodynamics, anaerobic benzoate biodegradation is accomplished by a syntrophic association of benzoate-degrading anaerobic strain(s) and hydrogen-utilizing anaerobes, which keep the partial pressure of hydrogen formed from aromatic ring biodegradation below 10^{-6} atmospheres. Complete 2CP biodegradation proceeds by dechlorination to phenol; biotransformation of phenol to benzoate, followed by benzoate biodegradation as described above. Benzoate is a central intermediate through which many aromatic compounds are channeled during anaerobic biodegradation. Thus, the complete biodegradation of 2CP or 3CB is accomplished by a cooperative anaerobic bacterial food chain, and the metal effect(s) observed may on a bacterial strain, or on a particular biodegradative activity.

We investigated the effect of added Cu(II), Cd(II), Cr(VI), and Hg(II) on the biodegradative activities of four anaerobic bacterial consortia which performed all or part of the biodegradative pathway involved in the food chain described above in a minimal anaerobic medium with low organic carbon and low biomass production. Two anaerobic consortia which degraded either 2CP or 3CB were examined for metal effects on dechlorination. Phenol and benzoate anaerobic bacterial consortia derived by adapting the 2CP consortium to phenol or benzoate as the target compound. After this adaptation the Phenol and Benzoate derivative consortia lost their ability to degrade 2CP and phenol, respectively, and were used to investigate the effect of added Cu(II), Cd(II), Cr(VI), and Hg(II) on intermediate steps in complete biodegradation of 2CP and 3CB, i.e. phenol biotransformation and biodegradation of benzoate.

Initially, we exposed the four anaerobic bacterial consortia to a wide range of Cu(II), Cd(II), Cr(VI), and Hg(II)concentrations, i.e. 0.01-100 ppm, and monitored biodegradation of the target compound to determine the concentration at which the initial biodegradative step did not occur, i.e. complete inhibition of biodegradation,; to determine a concentration at which the initial biodegradative step proceeded at approximately 50% of control consortia; and the highest concentration at which no metal effect was observed. In this initial study, samples from the four consortia were analyzed by HPLC to monitor dechlorination and/or aromatic biodegradation. A 50% inhibition concentration was established, and experiments were done to determine the relative sensitivities of the individual biodegradation steps, i.e. loss of parent compound; formation and loss of intermediates (phenol, benzoate,

volatile fatty acids); and formation of the endproduct, methane (CH_4), to a sublethal concentration of each added metal ion.

Data obtained with added Hg(II) suggested either adaptation to Hg(II), presence of Hg(II)resistant anaerobic bacterial strains, and/or mercury biotransformation was further investigated by splitting the apparently Hg(II) resistant culture and determining if the resistance to Hg(II) was biotic or abiotic.

To determine the potential to use these consortia, or similar anaerobic bacterial strains, for bioremediation in estuarine or marine environments, their tolerance to salinity was also determined.

ACCOMPLISHMENTS: The effect of 0.01-100 ppm metal ion concentrations on biodegradation rates of the four target compounds was determined. None of the target compounds were biodegraded @ >5-100 ppm of any metal ion tested. Cd(II), Cu(II), and Cr(VI) had no effect on biodegradation @ ≤0.1ppm. Hg(II) inhibited 2CP and phenol biodegradation @ 0.1ppm, but had no effect on 3CB or benzoate biodegradation at this concentration. Cd(II) prevented biodegradation of 2CP, phenol and benzoate @ ≥2ppm, while 3CB dechlorination was not observed @ ≥0.5ppm. Cr(VI) prevented biodegradation of 2CP and phenol @ ≥2ppm, benzoate biodegradation @ ≥1ppm, and 3CB dechlorination @ ≥ 5ppm. Hg(II) prevented 2CP and 3CB dechlorination @ ≥5ppm, phenol biodegradation @ ≥1ppm, and benzoate biodegradation @ ≥2ppm. Cd(II) decreased 2CP, phenol and benzoate biodegradation rates between 0.5-1.0 ppm; and 3CB dechlorination rates between 0.1-0.5 ppm Cd(II). Cr(VI) decreased 2CP, phenol and benzoate biodegradation rates between 0.5-2.0 ppm; and 3CB dechlorination rates between 0.1-0.5 ppm Cr(VI). Cu(II) decreased 2CP, phenol and benzoate biodegradation rates between 0.5-1.0 ppm; and 3CB dechlorination rates between 1.0-2.0 ppm. Hg(II) reduced 2CP dechlorination rates between 0.1-1.0 ppm; phenol biodegradation @ 0.1-0.7 ppm; benzoate biodegradation @ 1.0-2.0 ppm; and 3CB dechlorination rates @ 2.0ppm.

Sublethal inhibition studies were conducted at metal ion concentrations (Table 1) that reduced biodegradation rates of 2CP, phenol, benzoate and 3CB approximately 50% to study the effect of these metal ions on formation and biodegradation of intermediates.

Table 1. Sublethal concentrations of metal ions used.

Metal ion	Consortium and target compound			
	2CP	Phenol	Benzoate	3CB
Cd(II)	0.8[a]/7.1[b]	0.8/7.1	1.0/8.9	0.3/2.7
Cr(VI)	2.5/48.1	1.0/19.2	1.7/32.7	0.3/5.8
Cu(II)	1.2/18.9	0.5/7.9	0.6/9.4	1.0/15.7
Hg(II)	0.3/1.5	0.7/3.5	1.0/5.0	1.5/7.5

[A]ppm; [b]umoles/liter

In 2CP consortia, an acclimation period (20 days), i.e initial period during which biodegradation is not detected, was observed with Hg(II) only. The phenol dechlorination product accumulated stoichiometrically in both controls and metal-amended consortia for 40-50 days, but declined thereafter. Benzoate was not detected in 2CP consortia under these conditions. Acetate, produced from aromatic biodegradation, was detected at somewhat elevated levels in metal-amended consortia. Methane production was 29-65% of controls with Cd(II), Cr(VI), and Cu(II), but was not affected by Hg(II), i.e 94% of controls. Acclimation periods for phenol biodegradation in the Phenol

consortium were only observed with Cu(II) and Hg(II), i.e. 13 and 20 days, respectively, but initial rates with Cd(II) and Cr(VI) were slower than controls. Benzoate was not detected as an intermediate in these Phenol consortia, indicating rapid benzoate biodegradation at these metal ion concentrations. Acetate was detected at higher concentrations than controls with all metal ions, with highest concentrations in Cu(II)-amended consortia. Methane production was suppressed 21-52% by these sublethal metal ion concentrations. Acclimation periods in Benzoate consortia were 7-21 days in metal-amended consortia. Hg(II) strongly inhibited methane production, reducing it by 97%, while the Cr(VI) sublethal concentration had no effect. Cu(II) and Cd(II) inhibited methane production 63 and 76%, respectively.

During the initial experiment in which 0.01-100 ppm added metal was tested, we observed Hg(II) adaptation, i.e. an extended lag phase with no degradation in the presence of Hg(II) followed by onset of rapid degradation of the target compound. At 1-2ppm Hg(II), 2CP and 3CB did not decline for 2 weeks, but within the subsequent 6 and 9 days, respectively, were completely degraded. This adaptation phenomenon was confirmed by repeating the 2CP and 3CB experiment with 2 ppm Hg(II). The Hg(II)-adapted 3CB consortia was then used to further investigate the nature of the adaptation phenomenon. The Hg(II)-adapted 3CB consortia were split with one-half being passed to fresh media containing 3CB + 2 ppm Hg(II), and the second half being passed to fresh media containing only 3CB. A 50% transfer of a 3CB control consortium to fresh media was also prepared. 3CB biodegradation was initiated immediately in all consortia. The rate of 3CB biodegradation in the split Hg(II)-adapted 3CB consortia with and without additional Hg(II) was the same as the 3CB biodegradation rate in the control consortia. These data indicate that the Hg(II) adaptation phenomenon was not just a result of interaction between Hg(II) and medium components, but was an induced, transferable activity such as Hg resistance. We are currently attempting to isolate Hg-resistant anaerobic bacterial strains from the Hg(II)-adapted 3CB consortium to investigate the mechanism for Hg-resistance.

Effect of increasing salinity on biodegradation of the target parent compound by the four consortia in the absence of added metals was determined. Consortia were exposed to 5-30 ppt salinity by increments of 5ppt. 2CP dechlorination and subsequent transformation of phenol to benzoate in the 2CP consortium was rapid @≤20 ppt, but was much slower (10% of controls) @ 25 and 30 ppt. The Phenol consortium transformed phenol at rates similar to controls in consortia up to 10 ppt. Rates declined by 68% @ 15 ppt, and were 20% of control rates @ 20 and 30 ppt. Benzoate formed from phenol in this consortium was rapidly degraded in media up to 15 ppt, but was degraded very slowly at higher salinities. In contrast, benzoate biodegradation in the Benzoate consortium was unaffected up to 20 ppt, but did not occur @ 25 and 30 ppt. 3CB dechlorination was most sensitive to increasing salinity. At 5 and 10 ppt, dechlorination rates declined by 39% and 86%, respectively. Dechlorination of 3CB was only 10% of controls @ 15-25 ppt and was not observed by 135 days @ 30 ppt.

We quantified Cr(VI) versus Cr(II) in our anaerobic medium. Using the diphenylcarbazide method in the absence of $KMnO_4$ allowed us to quantify Cr(VI). Knowing the concentration of Cr(VI) we added, we were able to conclude that the reducing conditions in our medium resulted in Cr(VI) being reduced to Cr(III). Thus, the data we obtained above likely reflect sensitivity to Cr(III). Reducing conditions in the environment would cause a similar reduction of Cr(VI). We also modified the Bathocuproine method for Cu analysis to quantify Cu(I) vs. Cu(II). We performed the assay under an anaerobic gas phase in sealed serum tubes with and without hydroxylamine and found that the reducing conditions in our medium caused a portion of the Cu(II) to be reduced to

Cu(I). We also investigated the effect of added metal ions on the Eh of the medium to see if there was a correlation between observed inhibitory effects and initial Eh. Initial Eh increased with added metal ions, but there was no direct correlation between initial Eh and onset of inhibition by added metal ions.

CONCLUSIONS: Added Cd(II) and Cr(VI) inhibited 3CB dechlorination most strongly, while 2CP dechlorination was most strongly affected by Hg(II). Cu(II)inhibited benzoate biodegradation most strongly, while Hg(II) inhibited phenol biodegradation most strongly. Utilization of the acetate intermediate was most sensitive to Cu(II) and Hg(II). Production of methane, an endproduct of anaerobic biodegradation, was most sensitive to Hg(II). 2CP and 3CB consortia adapted to the presence of Hg(II) by a inducible, transferable, biological activity which is under further investigation. The conditions in our anaerobic medium reduced all of the Cr(VI) to Cr(III), and a portion of Cu(II) to Cu(I). There was no direct correlation between the Eh created by adding metal salts to our anaerobic medium and observed inhibition of target compound biodegradation. Inhibition of target compound biodegradation did correlate with the amount of a particular metal ion indicating an effect by the particular metal and not by general medium conditions.

SIGNIFICANCE: The added metal ion concentrations which inhibit specific steps in the anaerobic biodegradation of haloaromatic compounds under various environmental conditions will provide information on the impact that metal pollution may have on natural and bioremediative microbial processes. Bioremediation of environmental sites containing organic pollutants must be characterized for metal co-contaminants before bioremediation is initiated to determine the potential for inhibition of bioremediation processes by co-contaminating metals. Our data suggests that anaerobic metal resistance mechanisms may exist which could be used to bioremediate heavy metal pollution, or alleviate metal inhibition of organic pollutant bioremediation.

PATENT INFORMATION: No patents have been filed.

AWARD INFORMATION: Chun-Wei Kuo (Master's Degree Graduate Student) was awarded an American Society for Microbiology Travel Scholarship in 1995 for travel to the Annual Meeting in Washington, DC. Chun-Wei Kuo was awarded his Master of Science in May, 1996. His Master's Thesis was based on the data he generated in this research project.

PUBLICATIONS AND ABSTRACTS: (for the total period of the grant)

1. Effect of Heavy Metal Ions on the Biodegradation of Halogenated and Non-halogenated Aromatic Compounds in Anaerobic Bacterial Consortia. C-W Kuo* and B.R. Sharak Genthner. ASM Abstracts. 1995.

2. C-W Kuo* and B.R. Sharak Genthner. 1996. Effect of Added Heavy Metal Ions on Biotransformation and Biodegradation of 2-Chlorophenol and 3-Chlorobenzoate in Anaerobic Bacterial Consortia. Appl. Environ. Microbiol. 62:2317-2323.

3. B.R. Sharak Genthner, H. Beck and C-W. Kuo. Mercury Adaptation in a 3-Chlorobenzoate-degrading Anaerobic Consortium. (In preparation)