Journal of Hazardous Materials, 10 (1985) 141—147
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

EPA **Project Summary**

Biological Countermeasures for the Control of Hazardous Material Spills

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A study was conducted to investigate the feasibility of using microbiological processes to mitigate hazardous material spills in watercourses. A literature search, screening tests, laboratory-scale tests, and small-scale, simulated spill tests were conducted. Objectives were to (1) identify microorganisms capable of degrading selected hazardous materials, (2) identify their growth requirements and environmental factors affecting them, (3) determine the fate of selected hazardous materials in water, sediment, and biota, (4) develop methods for production, storage, reculture, and deployment, (5) investigate response requirements, and (6) evaluate the feasibility of biological countermeasures.

Study results showed that microorganisms can effectively remove certain hazardous materials since most significant ones are biodegradable. Potentially harmful secondary effects should be minor since microoganisms are a natural part of the aquatic environment. Furthermore, pathogenic bacteria are not likely to constitute a significant part (if any) of the countermeasure, and noxious sludges should not persist because the microorganisms should oxidize themselves following consumption of the hazardous material.

This Project Summary was developed by EPA's Municipal Environmental Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The possibility of accidental spills of hazardous substances poses a constant threat to the surface waters of the nation. Effective ways to control such spills and to mitigate their effects include physical and chemical techniques; biological countermeasures have not been thoroughly investigated to date. The attractiveness of a biological countermeasure for toxic material spills is twofold: (a) bacteria are natural components of ecological systems, and their use as a countermeasure does not constitute the introduction of a foreign material; and (b) bacteria can, under optimum conditions, metabolize organic hazardous materials

This material was originally published by the U.S. Environmental Protection Agency as EPA-600/S2-84-017.

to the principal end products, carbon dioxide and water. But because the use of a biological countermeasure imposes special constraints, its use must be carefully considered. To understand these special constraints, consideration must be given to the requirements of a general countermeasure and to the information needed to judge the suitability of a biological countermeausre.

The objective of this study was to investigate the feasibility of using microbiological processes to mitigate hazardous material spills in watercourses. Toward that objective, a literature search, screening tests, laboratory-scale tests, and small-scale, simulated spill tests were conducted to:

- identify candidate microorganisms capable of degrading selected hazardous materials;
- determine growth requirements and environmental factors affecting the growth of microorganisms that were found to break down hazardous materials successfully;
- determine the fate of selected hazardous materials, and of their breakdown products, in water, sediment, and biota;
- develop production, storage, reculture, and deployment methods;
- investigate response requirements; and
- evaluate the practical feasibility of biological countermesures.

Study Description

To begin the biodegradability studies, a list of 14 hazardous material candidates was developed. A preliminary review of the physical and chemical characteristics of these materials revealed that several compounds are quite soluble in water and would be dispersed by natural mixing processes upon spillage, whereas some, like xylene and benzene, are relatively insoluble but rapidly sorb to suspended particles and thus would remain in suspension. For experimental purposes, the list included a variety of hazardous materials because of their physical or chemical nature after spillage. Likewise, the chemical structures of the compounds were variable enough to provide an adequate test of biological countermeasures on these representative materials.

The final selection of hazardous materials was made after a literature review and initial screening tests. The purpose of the literature review was to gather information on the candidate materials regarding (1) physical and chemical characteristics of the most likely "spilled" chemicals, (2) previous biodegradation and biological treatment investigations, and (3) toxicity to organisms in fresh and marine waters. A summary of the toxicity and biodegradation information gathered from the literature review appears in Table 1.

Screening tests were performed for 11 hazardous materials to examine whether they were biodegradable and to determine their suitability for biodegradability measurement in a standard experimental test. These tests (Table 2) were conducted in aerated batch reactors seeded with municipal sewage sludge. Nitrogen, phosphorus, an alkalizing agent, and in some cases glucose and glutamic acid (as a supplemental food source) were added to the reactor along with the hazardous material. (Additions of glucose and glutamic acid were found to be unnecessary when acclimated sludge was used.) Total and volatile suspended solids (TSS and VSS), biochemical oxygen demand (BOD), pH, and total organic carbon (TOC) were monitored throughout the tests. In addition, microbiological samples were removed for analysis.

In the screening tests, the behavior of the hazardous material was sought as a contaminant in domestic sludge; therefore, disappearance of TOC was used to monitor removal (degradation or volatilization). Of the compounds studied, phenol, methanol, and nitrophenol appeared to be the most amenable to measurements related to biodegradation. Although acrylonitrile and nonyl phenol were also degraded by microorganisms, the reaction conditions were viewed as incompatible for large-scale field use. Because of volatility, isoprene, benzene, styrene, and xylene did not appear to be suitable

Table 1. *Biodegradability and Toxicity[1] of Hazardous Material Candidates*

Hazardous material	*Biodegradation shown?*	*Pathway known?*	*Toxicity defined?*
Acetone cyanohydrin	*Yes[2]*	*Partially*	*Yes*
Acrylonitrile	*Yes*	*Partially*	*Yes*
Aldrin	*Yes*	*Partially*	*Yes*
Benzene	*Yes*	*Yes*	*Yes*
Cyclic rodenticides and insecticides	*Yes[3]*	*Partially[3]*	*Yes*
DDT	*Yes*	*Partially*	*Yes*
Isoprene	*No[4]*	*No[4]*	*Partially*
Methanol	*Yes*	*Yes*	*Yes*
Nitrophenol	*Yes*	*Partially*	*Yes*
Nonyl phenol	*No*	*No*	*Partially*
Phenol	*Yes*	*Yes*	*Yes*
Styrene	*No[4]*	*No[4]*	*Partially*
Xylene	*Yes*	*Partially*	*Yes*
Toxaphene	*No*	*No*	*Yes*

[1]*The literature was reviewed to gather information on the physical/chemical characteristics of these compounds, previous biodegradation and biological treatment investigations, and the toxicity of the compounds to various organisms in fresh and marine waters.*
[2]*Following chemical dissociation.*
[3]*For a few compounds.*
[4]*No direct evidence, but should be biodegradable; probable pathway known.*

Table 2. *Screening Tests to Determine Biodegradability*

Hazardous material	*Volatilization demonstrated*	*Biodegradation demonstrated*	*Bacterial cultures*
Aldrin[1]			
Acrylonitrile		*X*[2]	Azotobacter vinelandii Clostridium pastereonin
Benzene	*X*		
Isoprene	*X*		
Methyl alcohol		*X*	Methanomonas methanica Pseudomonas *sp.*
Nitrophenol		*X*	Micrococcus *sp.* Pseudomonas *sp.*
Nonyl phenol		*X*	
Phenol		*X*	Pseudomonas *sp.*
Styrene	*X*		
Toxaphene[1]			
Xylene	*X*		

[1]*Results were inconclusive.*
[2]*Although biodegradation was demonstrated, it is not feasible for large-scale field applications.*

candidates for conclusive testing under the experimental conditions used in this study. Finally, the studies of aldrin and toxaphene were inconclusive because of sorption onto the reactor walls and analytical difficulties.

Following the screening tests, the three chemicals that appeared to be most amenable to measurements related to biological countermeasures (phenol, methanol, and p-nitrophenol) were subjected to treatability studies designed to delineate growth kinetic coefficients, the effects of environmental variables

(such as pH and temperature), and any other information necessary to conduct pilot-scale countermeasure tests. Based on these treatability tests, the following kinetic equations satisfactorily describe the bacterial growth and substrate removal kinetics:

$$\frac{dX}{dt} = -a\frac{dS}{dt} - k_d X \text{ and } \frac{dS}{dt} = -\frac{kXS}{K_s + S},$$

where:

X = biomass concentration, mg/l
S = substrate concentration, mg/l
t = time
a = cell yield coefficient (biological mass produced/substrate used)
k_d = cell decay coefficient, $time^{-1}$
K_s = Michaelis-Menten constant, mg/l (substrate concentration at which the substrate removal rate is one-half of the maximum rate)
k = substrate removal rate coefficient, $time^{-1}$

Table 3 summarizes the values of the parameters experimentally determined for phenol and methanol — the two materials selected for simulated spill tests.

Additional studies were conducted to determine the effects of a variety of experimental parameters, including temperature, pH, nutrients, and minerals, on bacterial growth and substrate removal.

Although the cell yield coefficient (a) and the Michaelis-Menten constant (K_s) did not change significantly with temperature, the substrate removal rate (k) was found to be temperature dependent for both methanol and phenol. But the modified Arrhenius equation, $k_{T2} = k_{T1}\theta^{(T2-T1)}$, commonly used to define the effect of temperature on substrate removal rate, did not accurately describe the temperature effect on k. The temperature coefficient, θ, changed with temperature range but fell between 1.0 and 1.4, depending on substrate, pH, salinity, and temperature. The substrate removal rate was also found to be dependent on pH. In phenol decomposition, which decreases pH, the buffering capacity of the system was important, whereas in methanol decomposition, which does not significantly affect pH, the initial pH of the water was an important factor.

The effects of trace element (Fe^{++}, Mg^{++}, Mn^{++}, Ca^{++}, and Zn^{++}) and nutrient (nitrogen and phosphorus) additions were also investigated. Addition of trace elements caused a slight increase in the phenol removal rate. The addition of nutrients, however, had no effect on phenol removal, but it did affect bacterial cell synthesis and decay. Though the cell yield coefficient (a) increased in the presence of nutrients, the cell decay

Table 3. Kinetic Parameters

	Hazardous material	
Parameter	*Phenol*	*Methanol*
K_s, *mg/l*	*236 ± 70*	*2330 ± 1410*
a, mg/l	*1.21 ± 0.06*	*1.25 ± 0.45*
k_d, $time^{-1}$	$0.066k^{0.87}$	$0.0115k^{0.634}$

Table 4. Oxygen Requirements for Substrate Removal and Bacterial Growth

Amount of oxygen used/g carbon for:	*Phenol*	*Methanol*
Complete substrate oxidation	*3.11g*	*4g*
Complete oxidation of the cell	*2.67g*	*2.67g*
Cell synthesis	*0.44*	*1.33*
	(3.11-2.67)	*(4-2.67)*

coefficient (k_d) decreased. In the methanol studies, neither nutrients nor trace elements had any effect on bacterial growth or substrate removal.

In both methanol and phenol studies, an initial lag phase was observed in the substrate removal rate. The average duration of the lag phase was roughly 4 and 9 hr for phenol and methanol, respectively. To compensate for the lag phase, supplemental aeration was required. Preliminary experiments indicated that the lag phase reflected a lag in bacterial growth rather than the decomposition of the substrate into intermediate compounds. The factors responsible for the lag phase were not determined, but the duration of the lag phase generally appeared to decrease with increasing temperature.

The oxygen requirements for biodegradation were also investigated. One gram of carbon is assumed to produce 1.88 g of organic cellular material, based on the approximate formulation of a bacterial cell ($C_5H_7NO_2$). Thus, based on the previously calculated cell yield coefficients, it was determined that 64.4% of the phenol and 66.5% of the methanol degraded was used for new cell synthesis. Given these assumptions, the oxygen requirements for substrate removal and bacterial growth could be calculated (Table 4).

Following the kinetic studies, simulated spill tests were conducted in aquaria and in ponds to investigate further the feasibility of using biological countermeasures. The results of these tests were used to develop experimental methods for large-scale biological countermeasure studies in a 30.5-m diameter, 3.0-m deep model lake. In addition, flowing-system tests were carried out in a model river.

Specific objectives of aquaria tests were to examine the hazardous material removal rates as a function of bacterial mass (i.e , the ratio of hazardous material mass to bacterial mass) and as a function of the addition of nutrient salts ($NaHCO_3$, KH_2PO_4, $(NH_4)_2SO_4$). In the phenol tests, a low phenol/bacteria ratio was desirable, though precautions had to be taken to ensure adequate dissolved oxygen levels. The substrate/bacteria mass ratio studies with methanol were inconclusive. For both substrates, the addition of nutrients (at levels determined from kinetic studies) caused roughly a two-fold increase in the biological decay rate. But doubling this nutrient concentration did not increase the decay rate perhaps because the nutrients reached a saturation level for bacterial growth.

Pond tests were designed to evaluate biological countermeasures in larger systems, and more specifically (1) to examine the effects of using acclimated as opposed to unacclimated bacteria, and (2) to evaluate the use of a portable treatment unit containing bacteria. These experiments confirmed the feasibility of the countermeasure with acclimated sludge, the possible use of unacclimated sludge, and the potential utility of a portable treatment unit.

In a model lake, dye tests were conducted to determine the dispersion rates and pattern of the hazardous material and to develop a method for calculating mass balance. The use of Rhodamine B dye was demonstrated to be a satisfactory method for establishing a dilution baseline and for tagging the hazardous material. In addition, the use of a barrier to contain the hazardous material and the biological countermeasure (both acclimated and unacclimated sludge) was investigated. A semi-rigid but maneuverable barrier was able to contain the spilled material successfully. As expected, the decomposition rate of phenol within the barrier was greater with the use of acclimated, rather than unacclimated, sludge.

A model river was used for material transport and bulk sludge application tests, studies of material exchange through cloth bags, and confining-barrier application tests. Based on these investigations, batch treatment systems were recommended over continuous-stirred-tank-reactor (CSTR) systems. The former are simpler to design and construct, require less aeration time, and produce more acclimated sludge than CSTR systems. For *in situ* treatment, application of bulk sludge was not an efficient

method for phenol and methanol because of sludge settling. Sludge-containing cloth bags were found to be a useful means of preventing dispersion of the spilled material.

Finally, it appears that the practicality of biological countermeasures using added organisms (as distinct from using indigenous organisms) will depend on the methods available for storage of the bacterial culture. Preliminary studies were conducted on freezing and on lyophilization of phenol-acclimated bacterial cultures. Though the feasibility of these storage techniques was demonstrated on a small scale, further work is needed to select appropriate storage and reconstitution methods, determine the shelf life of the stored material, evaluate the need for nutrient and mineral additions, and identify the amounts of materials that will be needed for a given spill (based on the chemical, the volume of the spill, etc.).

Conclusions

1. Based on criteria for assessing potential countermeasures for mitigating hazardous material spills, biological countermeasures appear useful because:
   a. Microorgnisms may be effective under some conditions for removing certain hazardous materials.
   b. Microorganisms that attack a variety of hazardous materials exist (e.g., the Pseudomonads).
   c. It should be possible to deploy microorganisms *in situ* or in a portable treatment system in a fresh liquid state, a powdered state, or a freshly reconstituted state.
   d. Potentially harmful secondary effects should be minor because microorganisms are a natural part of the aquatic environment. Pathogenic bacteria will not likely constitute a significant part, if any, of the countermeasure; noxious sludge should not be formed; and microorganisms should not persist, since they should metabolize their own protoplasm following consumption of the hazardous material and disappearance of the food source.

2. Based on the treatability tests, the following conclusions are made pertaining to the use of batch treatment systems as a countermeasure:
   a. Batch treatment systems are preferred over continuous-stirred tank-reactor (CSTR) systems for spills of phenol and methanol, especially when the spill concentrations are high. Batch systems require much less aeration time to achieve a certain effluent quality and produce more acclimated sludge than CSTR systems.
   b. Batch systems can be designed using numerical methods or using batch kinetic diagrams.
   c. Sludge-containing cloth bags were found useful for easy containment of the sludge when consecutive batch treatments were required. Floating cloth bags can be used for *in situ* treatment methods to prevent sludge settling.
   d. When sludge-containing bags are used for the removal of spills, the substrate removal rate by organisms can be expressed as:

$$\frac{ds}{dt} = -\frac{EkXS}{K_s+S}$$

   e. When sludge-containing cloth bags are used in a batch treatment system, the system can be designed in the same manner as a regular batch system except that Ek instead of k is used for the substrate removal rate coefficient. The aeration time required to achieve a given removal was observed to be slightly less than the theoretically computed time, probably owing to organisms that escaped from the cloth bags.

3. Based on spill control tests in a model river, the following conclusions can be made:
   a. Application of bulk sludge in streams is not an efficient method for phenol and methanol removal because of sludge settling. Floating cloth bags may be used to prevent this problem; however, this method is highly restricted by the reaeration capacity of streams and the

large amount of acclimated sludge required.

b. Fixed, confining barriers may be used to prevent the dispersion of spills. Once pollutants are contained within barriers, they may be treated in a batch manner. Cloth bags may be employed when the mixing intensity is not sufficient for complete suspension of sludge. Oxygen need only be supplied within or near the cloth bags.

4. Based on model lake tests, the following conclusions can be made:

a. Phenol spills contained by a barrier may be removed using unacclimated sludge from a local activated sludge domestic waste treatment plant.

b. Use of biological countermeasures will result in a significant impact on the dissolved oxygen resources in the aquatic system. However, this impact can be reduced by mechanical aeration.

Recommendations

The study recommends continuing development of biological countermeasures and a major research emphasis on the following items:

1. Studies on countermeasure storage and reconstitution to determine the shelf-life of the stored material, the need for additions of mineral salts, and the amount of material needed for spills of a given chemical;
2. Development of techniques for countermeasure application in quiescent and flowing systems;
3. Determination of additional candidate chemicals for application of biological countermeasures; and
4. Further confirmation of methods for calculating amounts of the countermeasure needed for a given spill volume.

The full report was submitted in fulfillment of Grant No. R802207 by the University of Texas under the sponsorship of the U.S. Environmental Protection Agency.

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The complete report, entitled "Biological Countermeasures for the Control of Hazardous Material Spills," (Order No. PB 84-140 276; Cost: $22.00, subject to change) will be available only from:

National Technical Information Service
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