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# Effects of Pollutants on Microbial Activities in Estuarine Surface Films

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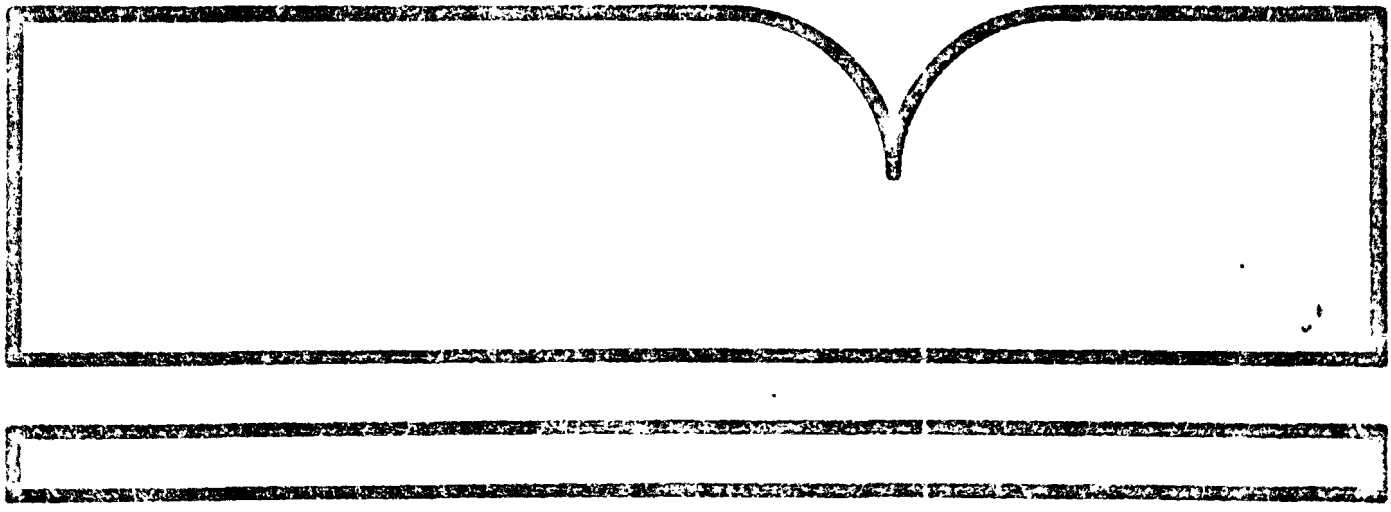

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# EFFECTS OF POLLUTANTS ON MICROBIAL ACTIVITIES IN ESTUARINE SURFACE FILMS

by

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|---|---|---|
| Microorganisms | Hydrocarbons | 06/F |
| Oil slick | Escambia Bay | 06/M |
| Pollutants | Hydrocarbonoclastic organisms | 06/T |
| Microcosms | Microbial Metabolic processes | |

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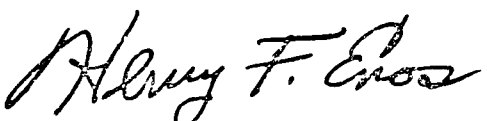
## FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities of organisms;
- the effects of toxic organics on ecosystem processes and components;
- the significance of chemical carcinogens in the estuarine and marine environments.

Research described in this report examines the fate of pesticides in estuarine surface layers. An understanding of the response of microbial populations to pollutants should aid in attempts to determine where toxic chemicals reside in the environment and to develop better methods to assess effects of such chemicals on biological processes.



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ABSTRACT

Samples of inshore surface films from Escambia Bay, Florida and from sites in the North Sea yielded populations of aerobic, heterotrophic microorganisms up to $10^8$ $ml^{-1}$ or $10^6$ $cm^{-2}$. Hydrocarbonoclastic organisms occured in relatively low populations. A comparison of species of yeasts prevalent in North Sea waters before and after oil production activities indicated a shift to a more widespread distribution of hydrocarbonoclastic forms with possible inhibition of a non-hydrocarbon utilizing species. Examination of various hydrocarbons and chlorinated compounds with the potential of being sequestered in natural films indicated that 66% could potentially alter microbial metabolic processes in the slick. In microcosm studies of estuarine systems, representative compounds demonstrated a selective effect for microfungi.

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SECTION I

## Introduction

Surface films on natural waters have been shown to contain high concentrations of organic carbon, nitrogen and phosphorous (William 1967), alkanes and chlorinated hydrocarbons (Seba and Corcoran 1969, Ledet and Laseter 1974). This organically enriched microhabitat also has been shown to contain high densities of bacteria relative to underlying waters. Sieburth (1965) reported bacterial populations up to $4 \times 10^4$ $ml^{-1}$ in surface films. The predominant bacteria were pseudomonads which expressed lipolytic activity. Harver (1966) found that bacteria, small algae, and colorless flagellates were concentrated in the upper 60 μm of surface water. In the studies of Crow et al. (1975), samples of the upper 10 μm of inshore surface films obtained by adsorption to membranes yielded microbial populations up to $10^8$ $ml^{-1}$ or $10^5$ $cm^{-2}$. These populations were typically 10 to 100 times greater than those in underlying waters at a depth of 10 cm. Predominant bacteria in the films were motile, nonpigmented, gram-negative rods. Colony-forming units of yeasts and molds were found in concentrations to $10^4$ $ml^{-1}$ or 28 $cm^{-2}$. The predominant species in the surface films were proteolytic and amylolytic,but exhibited only weak to negligible hydrocarbonoclastic and lipolytic activities.

Various researchers have reported the accumulation of various pesticides and polychlorinated aromatics in surface films. The bacterial bioconcentration of chlorinated hydrocarbon insecticides from aqueous systems appears to be a commonly occurring phenomenon (Grimes and Morrison 1975). Such binding of pesticides to cells suggests that the

presence of chlorinated hydrocarbons in surface films (Seba and Corcoran 1969) may be related in part to their microbial densities.

In other studies (Smith et al. 1975), heptachlor was shown to enhance or inhibit hexadecane utilization by Candida maltosa (from a freshwater oil slick) dependent upon aeration and pesticide concentration. The heptachlor in these culture systems appeared to be bound to the cells, but not metabolized. Walker and Cooney (1975) found stimulation of oxidation by hexadecane by Cladosporium resinae in the presence of non-utilizable substrates.

The alteration of microbial ecosystems in estuarine habitats by crude oil has been reported (Crow et al. 1975, Hood et al. 1975) and inhibition of estuarine bacteria by PCB formulations is known to occur (Bourquin and Cassidy 1975, Bourquin et al. 1975). Potential alteration of nutrient cycling in coastal areas mediated through hydrocarbon pollution will be of greater concern with the advent of the superports, development of offshore drilling along the eastern coast, and production from the Campeche Bay area in the Gulf of Mexico. Since PCB's, chlorinated pesticides, and detergent molecules are preferentailly soluble in or bound to hydrocarbons, the potential of an altered surface-film microflora with chronic oil pollution can be expected to increase.

The induction of bio-alteration by recalcitrant molecules is frequently unobserved in studies of macroscopic organisms. Unfortunately, long-term detrimental effects of pollutants usually are seen too late to prevent environmental damage. The rapid generation of bacteria and their metabolic responsiveness obviates some of these difficulties. Microorganisms, primary decomposers in the food web, can reflect potential deleterious environmental effects within a time span of days. Knowledge of the basic microecology of

estuarine surface films, particularly as affected by recalcitrant pollutants, may permit predictions of impending ecological stress of higher living forms.

The objective of this research was to examine the effects of hydrocarbons, pesticides and chlorinated biphenyls on the species composition and physiology of predominant groups of micororganisms in estuarine and marine surface films. Three basic approaches were taken to achieve the objective: (1) heterotrophic microorganisms were isolated from natural and man-mediated estuarine and oceanic surface films; representative isolates were examined for their interactions with select compounds; (2) selected pesticides, chlorinated biphenyls and polynuclear aromatic hydrocarbons were screened for their potential mutagenic and inhibitory capacity for microorganisms.

## SECTION 2

## Conclusions

Various hydrocarbons from polluting crude oil have the potential to alter the microbial composition of estuarine and oceanic surface slicks. Chlorinated aromatic compounds and various pesticides which have been shown to be sequestered in surface films may further affect microbial activities. Microcosm laboratory studies may be employed to show the selective effect of pesticides on microbial populations in estuarine habitats.

## SECTION 3

## Recommendations

Laboratory microcosms should be established with materials from selected estuarine habitats of known microecology. These microcosms should be enriched with traces of natural nutrients (glucose, cellulose, amino acids, etc.) and the response (metabolism and populations) monitored (short-term) in the absence and presence of xenobiotic molecules. The results should be compared with findings from field studies of environments exposed to the same xenobiotic. A major purpose of these studies is to determine the shortest exposure time for significant results from laboratory microcosms.

SECTION 4

## Materials

### Areas Sampled

| | |
|---|---|
| Gulf Breeze, Florida | |
| Range Point salt marsh | 8 samples |
| Escambia Bay | 23 samples |
| North Sea | |
| 35 stations between 54'N, 8'E and 66° 40'N, 10°E | 70 samples |

### Materials and Sources

Polycarbonate membranes, Nucleopore Corporation, Pleasanton, California

Standard Media (prepared with 50 per cent seawater)

| | |
|---|---|
| Marine Agar 2216 | Tryptic Soy Agar |
| Mycological Agar | MOF Medium |
| Spirit Blue Agar | Bushnell Haas Broth |

Specialized media (listed below) were prepared according to the methods of *Colwell and Wiebe (1970) and Hankin and Anagnostakis (1975).*

Proteolytic Enumeration Media
Amylolytic Enumeration Media
Lipolytic Enumeration Media
Hydrocarbon Enumeration Media (1 per cent hexadecane in Bushnell-Haas broth)
Phosphatase Media
Basal Broth
  Yeast: YNB (Difco)
  Bacteria: Bushnell-Haas Broth

### Chemicals Studied

| | | |
|---|---|---|
| Aldrin | DDT mixed isomers | Heptachlor |
| Aroclor 1221 | DDT-O,P[1] | Heptachlor epoxide |
| Aroclor 1242 | | Hexachlorobenzene |
| Aroclor 1260 | DDT-P[1],P[1] | 1-Hydroxychlordene |
| BHC | Diazinon | Malathion |
| Bux | Dichlone | Methyl Oxychlor |
| Captafor | Dicofol | Methyl Parathion |
| Captan | Dieldrin | Mirex |
| Carbaryl | Endosulfan | Pentachlorophenol (PCP) |
| Chlordane | Endrin | Trans Nonachlor |
| Chlordene | Halowax 1000 | Tetrachlorophenol |
| | Halowax 1051 | Toxaphene |
| DDD mixed isomers | Halowax 1099 | |

SECTION 5

## Experimental Procedures

### Environmental Sampling

The surface slick samples from Escambia Bay and the North Sea were collected with sterile polycarbonate membranes according to previously described procedures (Crow et al. 1975; 1976; 1977). In brief, sterile polycarbonate membranes were floated on the water. The membrane and adhering surface film were retrieved with either a sterile plastic dish or bucket which was submerged under the membrane and underlying waters or, in calm waters, by directly retrieving the membrane with a sterile forceps from the water surface. The membranes were placed into bottles containing sterile seawater or placed directly onto a solid nutrient medium.

Samples from the Amoco Cadiz spill included: a viscous, brown-black crude with low water of emulsion, a brown mousse, and surface film collected from tidal pools. Samples of each were collected in sterile 10 ml vials from various sites within 10 km north of the Portsal harbor.

### Laboratory Studies

Bottles containing membranes were returned to the laboratory under refrigeration and processed within an hour of collection. The bottles were agitated for 3 min on a wrist-action shaker. Aliquots were serially diluted and 0.1 ml of dilutions plated onto appropriate media. Bacteria were characterized physiologically with media prepared according to the formula of Colwell and Wiebe (1970). Proteolysis was determined with 2.0% skim milk and 0.1% yeast extract in 1.7% agar and with Thioglycollate gelatin medium (Difco) prepared with artificial seawater. Oxidative or fermentative carbohydrate metabolism was determined with Hof medium (Difco). Lipase and urease

activities were determined on Spirit Blue agar (Difco) and Urea agar (Difco), respectively. Both were prepared with artificial seawater at 20 $^{0}/_{00}$ salinity Yeasts and filamentous fungi were identified and examined for their capacity to grow on various hydrocarbons according to described procedures (Ahearn et al. 1971; Crow and Ahearn, 1979).

The Amoco Cadiz samples were held at room temperature and periodically over a 6-month period, 0.1 ml was cultured in 20 ml of enrichment broths and 0.1 ml was diluted 1:10 in a .1% Tween 80 sea water solution and inoculated onto Buschnell-Haas agar with .01% hexadecane, mycological agar prepared with sea water and marine agar. The enrichment broths were: filtered sea water with a 0.01% yeast extract, and sea water with .07% $(NH_4)_2$ $SO_4$ and sea water with both the $(NH_4)_2$ $SO_4$ and yeast extract. The enrichment broths were incubated at 20°C for up to 14 days and 0.01 ml samples inoculated onto the isolation agars by spread plate procedure every 3-4 days. Representative bacteria and fungi from the various selective media were characterized for their interactions with various pesticides and oil constituents. The mutagenic and inhibitory effect of selected pesticides, chlorinated biphenyls and polynuclear aromatic hydrocarbons was established using the tester strains developed by Ames et al. (1975).

The effects of selected pesticides on fungal development were examined using microcosms (Pritchard et al. 1979). A sediment-water test system contained 50 g of sand and detritus with 250 ml water (10-17 ppt salinity) from the Range Point salt marsh. This system was maintained at room temperature (23-25°C) with the water fraction aerated with a bubbler tube. The other microcosm, a continuous flow system, contained 144 g of sand, 250 ml detritus and 250 ml of water from the Range Point salt marsh

layered into 1000 ml growth vessels. The temperature was maintained at 23°C, salinity at 12-14 ppt and air was provided at a rate of 30 cc/min.

Pentachlorophenol (UL $^{14}C$, Pathfinder Lab, Inc.) was introduced into the sediment-water test system at 140 µg/l. Carbaryl (Naphthyl-1-$^{14}C$, California Bionuclear) and methylparathion (2,6-$^{14}C$ ring-labeled, Amersham-Serala Corporation) were introduced into the continuous-flow microcosm from the reservoir at a rate of 14 ml/hr, giving a final concentration in the growth vessel of 214 µg/l for the former and 75 µg/l for the latter.

At approximately weekly intervals, 1.0 ml of water and detritus were removed from each growth vessel and from the headbox water. Detritus and water from the Range Point salt marsh also were examined weekly for fungi.

SECTION 6

## Results and Discussion

### Environmental Studies

Surface slick materials from Escambia Bay were plated on selective media to determine the prevalence of physiologic types (Table 1). For these samples, lipolytic and amylolytic organisms were more prevalent than proteolytic and hydrocarbonoclastic types.

Table 1. Heterotrophic Microbial Populations in Surface Slicks of Escambia Bay.

| Total Aerobic | No. Samples | # $CM^{-2}$ Range | Mean |
|---|---|---|---|
| Heterotrophs | 23* | 1-2.8 x $10^7$ | 1.24 x $10^6$ |
| Amylolytic | 21 | 1-5.8 x $10^7$ | 2.8 x $10^6$ |
| Proteolytic | 15 | 1-4.6 x $10^5$ | 3.2 x $10^4$ |
| Hydrocarbonoclastic | 8 | 1-2.7 x $10^4$ | 3.7 x $10^3$ |
| Lipolytic | 13 | 1-1.8 x $10^7$ | 1.4 x $10^6$ |
| Yeasts | 14 | 1-2.4 x $10^3$ | 1.3 x $10^2$ |

*No. samples positive of 23 total samples.

No yeasts with significant hydrocarbonoclastic activities were obtained. None of the samples, however, was from sites influenced by notable hydrocarbon pollution. The influence of oil production activities appeared to affect the composition of the surface film flora of the North Sea.

Species of yeasts from surface waters of the North Sea were compared with those isolated in an earlier study prior to the development of oil

production (Table 2). Candida guilliermondii, a hydrocarbonoclastic yeast, was obtained frequently in 1976, whereas only several atypical isolates initially identified as Candida sp. were obtained in 1964-66. In 1976, the incidence of Aureoobasidium pullulans appeared reduced. Meyers et al. (1968) found yeasts at all stations in 99% of 84 samples at cell densities between 35-50 cells/L with the maximum density of >3,000 cells/L. In 1976, yeasts were isolated from 100% of the surface samples collected at the 35 stations and from 28 of the 35 samples collected at 10 m. Yeast densities at the surface averaged 76 cells/L and 35 cells/L at 10 m.

Relatively few fungi were isolated from the Amoco Cadiz oil (Table 3). The direct sampling onto agar plates of all samples gave only a few colonies, indicating that fungal populations were <10 colony forming units per 100 ml that represented only a few species, but when the samples were vigorously agitated in a Tween 80 solution, densities in some samples ranged to nearly 50 cells/ml and yielded up to five different species. The greatest variety of species was obtained from the surface films. In comparison to surface films and water samples examined in earlier work, the fresh crude oil from the Amoco Cadiz appeared selective and possibly inhibitory to normal marine yeast flora. Certain volatile hydrocarbons, dependent upon concentration, may be lethal to yeasts (Ahearn et al. 1971). In preliminary tests, Iranian crude (about 30% naphthenes) proved inhibitory to representative isolates of Debaryomyces hansenii, in spot tests. This species is the most common yeast in North Sea waters. The presence of odorous volatile oil fractions at the shore adjacent to the wreck was quite noticeable, even 10 days after the spill. The high concentrations of these volatile fractions may have markedly reduced the densities and species of yeasts brought into contact with the oil.

Table 2. Yeasts from the North Sea

| 1964-66* | Incidence** | 1976 | Incidence |
|---|---|---|---|
| Debaromyces hansenii | 38 | D. hansenii | 46 |
| Rhodotorula rubra | 29 | Candida guilliermondii | 29 |
| Aureobasidium pullulans | 23 | Cryptococcus laurentii | 14 |
| Candida diddensii | 18 | Rhodotorula rubra | 11 |
| C. tropicalis | 12 | Cr. albidus | <10 |
| R. pilimanae | 11 | Cr. gastricus | <10 |
| Hanseniaspora uvarum | 10 | Torulopsis candida | <10 |
| C. zeylanoides | <10 | R. graminis | <10 |
| C. obtusa | <10 | R. lactosa | <10 |
| C. krusei | <10 | C. tropicalis | <10 |
| C. lipolytica | <10 | C. parapsilosis | <10 |
| C. silvicola | <10 | Kloeckera apiculata | <10 |
| C. tenuis | <10 | Rhodosporidium capitatum | <10 |
| Rhodosporidium infirmio-miniata | <10 | Sporobolomyces roseus | <10 |
| Rhodotorula gram | <10 | S. gracilis | <10 |
| Sporobolomyces roseus | <10 | S. albo-rusescens | <10 |
| Hanseniaspora californica | <10 | Aureobasidium pullulans | <10 |
| C. guilliermondii (Candida sp.) | <10 | | |
| Total Samples | 84 | | 70 |

*See Meyers et al. 1967
**Per cent occurrence in total samples

Table 3. Fungi Isolated from Amoco Cadiz Oil

| Species | Brown-Black Oil | Mousse | Surface Film |
|---|---|---|---|
| *Rhodotorula rubra* | + | + | + |
| *Debaryomyces hansenii* | - | + | + |
| *Candida tropicalis* | + | + | + |
| *C. lipolytica* | - | + | - |
| *C. guilliermondii* | + | + | + |
| *Aureobasidium pullulans* | - | - | + |
| *Penicillium* sp. | + | + | + |
| *Cladosporium* sp. | - | + | + |
| *Mucor* sp. | - | - | + |
| *Fusarium* sp. | + | + | + |

Laboratory Studies—Mutagenicity Tests

The Salmonella/mammalian-microsome mutagenicity test was used to detect compounds with potential of altering surface-slick flora (Table 4). More than 60% of the compounds increased the rates of mutagenicity of the salmonellae. Table 4 compares also the results of the current study with publications using the Salmonella/mammalian-microsome mutagenicity test. Shirasu et al. (1976) prescreened all the pesticides with the rec-assay system,using Bacillus subtilis and apparently some of the mutagens in our test were eliminated by their prescreening. Marshall et al. (1976) used Salmonella that were not as sensitive to mutagens as were the ones used in our test; thus,we report more mutagens. Three compounds, methyl parathion, carbaryl, and pentachlorophenol, were selected for further studies in microcosms.

Microcosm Studies

Changes in the microfungal populations of microcosms established from estuarine sediments and water of the Range Point salt marsh were monitored upon the addition of selected pesticides (Table 5).

Addition of these pesticides to the microcosms altered the pattern of species recovered. The prevalent fungi in the sediment of the carbaryl microcosm changed from Trichoderma to an overgrowth of Fusarium. In the methylparathion growth vessel,Trichoderma, initially predominant and was succeeded by a species of Penicillium. The Penicillium I, provisionally classified within the Penicillium chrysogenum series, was isolated in large numbers in the final six samplings. A different species, Penicillium II, provisionally classified within the Penicillium canescens series, predominated during the sampling period in the sediment of the pentachlorophenol sediment-water test system.

Table 4. Comparison of Salmonella/mammalian-microsome Mutagenicity Test Results with Published Reports Using the Same Test.

| Pesticide/Chemical | Mutagen[a] | Results of References |
|---|---|---|
| Aldrin | + | -(3) |
| BHC | + | -(3) |
| Bux | + | None |
| Captafol | + | -(3) |
| Captan | + | +(1),(2),(3) |
| Carbaryl | + | -(2),(3) |
| Chlordane | ± | None |
| Chlordene | - | None |
| DDD mixed isomers | + | None |
| DDT mixed isomers | + | -(2),(3) |
| DDT-$O_1$, $P_1$ | - | None |
| DDT-$P^1$,$P^1$ | ± | None |
| Diazinon | - | -(2),(3) |
| Dichlone | + | -(3) |
| Dicofol | + | None |
| Dieldrin | - | -(1),(2),(3) |
| Endosulfan | - | None |
| Endrin | - | None |
| Heptachlor | - | -(2),(3) |
| Heptachlor epoxide | - | -(2) |
| Hexachlorobenzene | - | None |
| 1-Hydroxychlordene | + | None |
| Malathion | - | -(3) |
| Methyl Oxychlor | + | None |
| Methyl parathion | - | None |
| Mirex | - | None |
| Trans nonachlor | ± | None |
| PCP | ± | -(3) |
| Tetrachlorophenol | + | None |
| Toxaphene | ± | None |
| Aroclor 1221 | + | None |
| Aroclor 1242 | ± | None |
| Aroclor 1260 | - | None |
| Halowax 1000 | + | None |
| Halowax 1051 | ± | None |
| Halowax 1099 | - | None |

[a] + = mutagen; - = nonmutagen

(1) McCann, J., E. Choi, E. Yamisoki, and B. Ames. 1975. Proc. Nat. Acad. Sci.

(2) Marshall, T. C., W. H. Dorough and H. E. Swim. 1976. J. Agric. Food Chem. 24:560-563.

(3) Shirasu, Y., M. Moriya, K. Kato, A. Furuhaski and T. Kada. 1976. Mutation Res. 40:19-30.

Table 5. Prevalent fungi isolated from salt marsh microecosystems after addition of selected pesticides.

| Pesticide | Microcosm Environment | Prevalent Fungi Initially Isolated | | | Prevalent Fungi Finally Isolated | | |
|---|---|---|---|---|---|---|---|
| | | Fungus | Frequency | Range | Fungus | Frequency | Range |
| Carbaryl | Water | Fusarium[1] | 3/7[2] | 80-TNC[3] | Fusarium | 6/8 | 30-340 |
| | Sediment | Trichoderma | 4/7 | 30-TNC | Fusarium | 6/8 | 20-350 |
| Methyl-parathion | Water | Cladosporium | 3/7 | 20-80 | Penicillium I | 3/8 | 10-20 |
| | Sediment | Trichoderma | 6/7 | 60-TNC | Penicillium I | 6/8 | 30-290 |
| Penta-chlorophenol | Water | Cladosporium | 3/7 | 20-120 | Penicillium II | 2/8 | 10-120 |
| | Sediment | Penicillium II | 5/7 | 40-950 | Penicillium II | 8/8 | 70-460 |

[1]Genus of most commonly isolated fungus.

[2]Number of times fungus isolated/number of weeks sampled.

[3]Range of number of fungi isolated from 1 ml of sample.

Labeled pesticide molecules were added to batch culture systems contained only sterile seawater or dilute broth and inoculated with the fungi (Table 6). The Fusarium isolated from the carbaryl microcosm slowly released traces of $^{14}CO_2$ at approximately the same rate from both batch cultures. Trichoderma sp. gave negligible release of $^{14}CO_2$. The release of $^{14}CO_2$ was most notable from PCP in the dilute broth culture. These low levels of $^{14}CO_2$ release suggest low level contaminant molecules as their source, but no such contamination was detected.

These preliminary studies indicate that pollutant pesticides may select for fungi and possibly alter the normal recycling of nutrients in microhabitats. Future studies should evaluate the use of such microecosystems to predict rates of biodegradation and the fate of xenobiotic molecules.

Table 6. Batch culture of selected fungi with $^{14}C$ labeled pesticides.

| Fungus | Pesticide | Culture Medium | Days to Maximum $^{14}CO_2$ Evolution | Per cent of Pesticide Molecules Degraded |
|---|---|---|---|---|
| Fusarium | Carbaryl | Dilute mycological broth | 10 | 0.45 |
| | | Seawater | 13 | 0.51 |
| Trichoderma | Carbaryl | Dilute mycological broth | 20 | 0.11 |
| | | Seawater | 20 | 0.01 |
| Penicillium I | Methyl-parathion | Dilute mycological broth | 17 | 0.25 |
| | | Seawater | 13 | 0.72 |
| Penicillium II | Pentachloro-phenol | Dilute mycological broth | 20 | 0.17 |
| | | Seawater | 20 | 1.74 |

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