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Ecological Research Series

MICROBIAL INTERACTIONS WITH PESTICIDES IN ESTUARINE SURFACE SLICKS

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Gulf Breeze, Florida 32561

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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.

Microorganisms are considered ultimate disposers of pollutants, and therefore microbial considerations are essential in the proper functioning, response, and recovery of environmental degradation of marine ecosystems. Information on microbial interactions in specific niches (air-sea interface) is necessary to assess the effects on both biodegradative potential and on microbiological activities after a pollutant reaches the estuarine environment. The air-sea interface is a unique membrane, vitally important to marine food chains and implicated in concentration and transport of pollutant chemicals. Studies such as this report can enhance our understanding of this unique ecotone and contribute significantly to valid environmental assessment of a pollutant's effect on the marine ecosystem.

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ABSTRACT

Estuarine surface films from Escambia Bay, Florida, and adjacent waters were sampled by using the membrane adsorption technique to enumerate microbial populations. Samples of the upper 10 μm of estuarine surface films yielded microbial populations up to 10^8 ml^{-1} or 10^5 cm^{-2}. These populations were 10 to 100 times greater than those in underlying waters at a depth of 10 cm. Predominant bacteria in surface films as isolated on Marine Agar (Difco) were motile, nonpigmented, gram-negative rods. Colony-forming units of yeasts and molds on Mycological Agar (Difco) prepared with 50 per cent seawater were found in concentrations to 10^4 ml^{-1} or 28 cm^{-2}. The predominant surface film isolates from Marine agar were proteolytic and amylolytic, but exhibited only weak to negligible hydrocarbonoclastic and lipolytic activities. A greater proportion of the surface-film bacteria, as compared to those at 10 cm depth, were capable of growth on freshwater media. With selective isolation media, amylolytic, and lipolytic bacteria appeared to comprise a more significant proportion of the total population. Twenty-one representative bacteria, yeasts, and filamentous fungi from initial sampling of surface microlayers were tested for the effects of selected pesticides on utilization of various substrates. No inhibition by heptachlor or methoxychlor was noted with glucose as a carbon source. One bacterium was sensitive to PCB formulations. In subsequent studies with 53 isolates representative of more diverse physiological groups, o-chlorophenol, 1-chloronaphthalene, PCB 1016, and pentachlorophenol were inhibitory to a large portion of the isolates. Heptachlor inhibited 2 isolates and methoxychlor inhibited only 1 isolate. In contrast, with hydrocarbon as a substrate, microorganisms were more frequently inhibited by various aromatic or chlorinated hydrocarbons. For example, heptachlor, biphenyl, pyrene, and PCB 1016 significantly reduced hexadecane utilization by representative surface film bacteria. In a few instances, trace concentrations of pesticides enhanced hydrocarbon utilization. In studies with yeasts, high concentrations of heptachlor appeared to have a slight stimulating effect on utilization of hexadecane by C. maltosa, but no effect with C. lipolytica. The complex nature of metabolic responses with varying substrate and pesticide indicates that multiple assay procedures are required to detect the altering capability of pesticides within the surface film ecosystem.

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SECTION 1

INTRODUCTION

The surface microlayer is a unique microbial habitat occurring at the air-water interface. Accumulations of surface active organic material in this region leads to production of calm areas in disturbed waters. Chemical and physical studies of these surface films indicate a wide range of organic and inorganic components (Baeir 1970, 1972; Ewing 1950, Garrett 1965, Sutcliffe et al. 1963). Several recent studies propose a significant role for the surface microlayer in environmental processes (MacIntyre 1973, Parker and Barsom 1970). Although numerous studies of the physical and chemical properties of surface films have been conducted, little is known of the biological activities and interactions occurring within the surface microlayer and surface film.

Investigators have reported extensive microbial populations associated with the surface region (Zobell 1946, Gunkel 1973, Parsons and Takahashi 1973). Coupled with these observations, the rich varied supply of organic and inorganic nutrients suggest a biologically active region.

Recent studies (Seba and Corcoran 1970, Harvey et al. 1973, Stadler and Ziebarth 1976) demonstrated the presence of certain chlorinated hydrocarbons in surface films. Others (Hartung and Klingler 1970, Sayler and Colwell 1976) have suggested that surface film materials will rapidly concentrate chlorinated hydrocarbons and aromatic hydrocarbons from aqueous systems. Accumulation of such compounds within the surface film may alter the physiological activity of microorganisms associated with natural surface films. Rapid sequestering of chlorinated hydrocarbons in accidentally spilled hydrophobic materials may alter response of an ecosystem to this stress, thus lengthening the residence time of these materials in the environment.

The potential for hydrocarbon pollution of coastal and estuarine waters is increasing. The role of surface microlayer microorganisms in the dispersal of accidental spills and the functions of populations within naturally occurring surface films are closely associated. An understanding of the bionomics of pollutant molecules is basic to the establishment of tolerable levels for pesticides, chlorinated hydrocarbons, and aromatic hydrocarbons in aquatic systems. Determination of baseline microbial populations of natural surface films, their response to the chemical and physical conditions, as well as the numerous interactions, are essential to this development.

The basic objectives of this research were: (1) to determine microbial population numbers and diversity in estuarine surface films, (2) to study the metabolic activity of surface film isolates on a wide range of substrates, (3) to determine the interactions of various pollutant molecules with

microbial metabolism of selected substrates.

To facilitate the presentation of this material, studies will be grouped into two categories.

Initial Studies: primarily dealing with establishing surface film microbial populations, with further evaluation of metabolic potential and pesticide interference on a single culture basis.

Studies of Selected Metabolic Types: evaluation of surface film microbial populations with emphasis on establishing numbers of major metabolic types (proteolytic, amylolytic, lipolytic, and hydrocarbonoclastic groups) and laboratory evaluation of pesticide interactions with these types.

SECTION 2

CONCLUSIONS

It is concluded from this study that estuarine and coastal surface slicks usually contain dense numbers of aerobic, heterotrophic microorganisms. No single enrichment medium depicts the true number or metabolic range of the microorganisms present in any single slick. The growth of representative surface slick microorganisms may be affected by the presence of certain aromatic or chlorinated organic molecules. The effects may stimulate metabolism of select compounds, (e.g., hexadecane) and concomittantly decrease recovery of recalcitrant molecules (e.g., naphthalene, biphenyl, heptachlor) from culture systems. Conversely, the growth or metabolism of certain surface slick flora may be inhibited by the presence of chlorinated aromatic compounds. It is suggested that the sequestering of recalcitrant molecules within surface films affects natural microbial processes.

SECTION 3

RECOMMENDATIONS

It is recommended that microbiological assay systems be developed for rapid quantitative measurement of specific toxicity by chlorinated aromatic compounds. These assays should be functional for a marine system and predict potential harm from industrial chemicals. Impairment of metabolism, inhibition of growth, and alteration of the genome should be considered as primary indices for evaluation.

SECTION 4

MATERIALS

Areas Sampled

Barataria Bay, Louisiana	
Airplane Lake	2 samples
Bayou Fer Blanc	2 samples
Gulf Breeze, Florida	
Cove adjacent to EPA Laboratory	13 samples
Waste Pond EPA Laboratory	10 samples
Sewage Outfall Pensacola	2 samples
Escambia Bay Mid Bay near Channel	4 samples
Boat Slip EPA Laboratory	2 samples
Range Point	8 samples
Bayou Chico	6 samples
	49 samples

Materials and Sources

Polycarbonate membranes, Nuclepore Corporation, Pleasanton, California

Standard Media (prepared with 50 per cent seawater)
- Marine Agar 2216
- Mycological Agar
- Spirit Blue Agar
- Tryptic Soy Agar
- MOF Medium
- 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 and Sources

^{14}C-heptachlor, Velsicol Chemical
PCB Formulations, Monsanto Chemical
cyclohexane, Fisher Scientific
methylcyclohexane, Fisher Scientific
ethyl benzene, Eastman Organic Chemicals
naphthalene, Matheson, Coleman and Bell
biphenyl, Eastman Organic Chemicals
tetradecane, Eastman Organic Chemicals
hexadecane, Eastman Organic Chemicals
eicosane, Sigma Chemical Company
mirex, Chem Services
1-chloronaphthalene, Aldrich Chemical
o-chlorophenol, Aldrich Chemical
endrin, Chem Services
methoxychlor, Chem Services
pyrene, Fisher Scientific
pentachlorophenol, Matheson, Coleman, and Bell
anthracene, Matheson, Coleman, and Bell

SECTION 5

EXPERIMENTAL PROCEDURES

ENVIRONMENTAL SAMPLING

Microbiological

Initial studies lead to the development of a microbiological surface slick sampling technique (Crow et al. 1973). The polycarbonate membrane (Nuclepore Corporation) had several suitable characters for surface sampling. The polycarbonate membranes have a very low density and are thus capable of floating even when saturated with water. Membranes composed of other materials (cellulose acetate, etc.) were found to sink rapidly when saturated with water. As compared to screen samplers, the membranes are easier to manipulate and problems with sterilization of sufficient samplers are non-existant. The membrane (47 mm dia, 0.4 μm pore size) absorbs approximately 50 μl of surface film permitting collection of a 20 to 40 μm thick sample. The size of a sample, however, will vary with the nature and thickness of the slick, a phenomenon common to all surface slick samplers.

The sterile polycarbonate membranes were floated on the water surface. The membrane and adhering surface film were retrieved with either a sterile plastic dish 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 100-ml bottles containing sterile seawater or placed directly onto a nutrient agar medium. Upon return to the laboratory, 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. In initial studies, dominant colonial types were picked from the enumeration medium, subcultured to assure purity, then placed on a maintenance medium subsequent to their physiological characterization.

In more recent studies, selective isolation media were used to detect major physiological groups. Serial dilutions of the membrane rinse water were plated onto media designed to detect proteolytic, amylolytic, lipolytic, and hydrocarbonoclastic microorganisms (Colwell and Wiebe 1970; Hankin and Anagnostakis 1975). Predominant organisms from each physiological group were isolated and stored on the appropriate stock culture medium.

Chemical

Large polycarbonate membranes (29 cm d, 0.4 μm pore size) were used to sample surface slicks. The membranes were retrieved from the water surface

and placed in 50 ml of petroleum ether. The petroleum ether extracts were concentrated and analyzed by Gas-Liquid chromatography with electron capture detector.

LABORATORY STUDIES

Initial Study

The physiological patterns of the predominant aerobic heterotrophs (41 cultures) from initial isolation medium (Marine Agar 2216) were established by using methods described by Colwell and Wiebe 1970; Hankin and Anagnostakis 1975. Effects of heptachlor, methoxychlor, and PCB formulations (aroclor 1016, 1242) on growth in Marine Agar were determined with a modified antibiotic sensitivity test. Selected strains were tested for the effect on ethanol utilization with heptachlor (up to 100 μg/ml) and methoxychlor (up to 200 μg/ml) fortified with 2 per cent ethanol.

Hydrocarbon metabolism of 2 yeasts, *Candida maltosa* and *C. lipolytica*, was studied by using a YNB-broth supplemented with hexadecane. Comparisons were made between systems containing hexadecane alone, hexadecane and heptachlor, hexadecane and methoxychlor, and hexadecane with the addition of glucose and glycerol.

To determine if heptachlor was being metabolized by *C. maltosa*, the CO_2 generated during the metabolism of a hexadecane and 14-C-heptachlor substrate was monitored. Cultures were extracted with petroleum ether. The petroleum ether fraction was separated and washed with water. Cells were centrifuged from the culture fluid and PE extracts and all fractions were assayed for 14-C-heptachlor.

The uptake of constituents of a synthetic crude oil, in the presence of selected carbohydrates and pesticides, (Table 1) by *C. lipolytica* and *C. maltosa* in a YNB basal broth was evaluated. Substrate combinations tested included: synthetic crude, synthetic crude and heptachlor, synthetic crude and methoxychlor, synthetic crude and glucose, synthetic crude and glycerol.

Studies of Selected Metabolic Types

Representative isolates of the major physiological types of microorganisms isolated from surface slicks were screened for inhibition by aromatic and chlorinated hydrocarbons (Table 2). Organisms were grown in Marine broth or Mycological broth for 48 hr. A minimal inoculum (.1 ml) was then added to the appropriate nutrient medium containing 100 ppm, 10 ppm, or 1 ppm of aromatic or chlorinated hydrocarbon dissolved in acetone. All cultures were initially screened for sensitivity to acetone. Inhibition was determined by a reduction of optical density compared to the acetone control. Hexadecane utilizing bacteria and a series of 5 nitroso-guanidine induced mutants of *C. lipolytica* were tested for reduction of hexadecane utilization in the presence of chlorinated and aromatic hydrocarbons.

Model hydrocarbon (hexadecane) surface slicks were exposed to water

TABLE 1. SYNTHETIC CRUDE OIL COMPOSITION

Item	Amount	Weight (gm)	Moles	Mole Ratio to Hexadecane
Cyclohexane	1.2 ml	.94	.011	.39
Methylcyclohexane	2.6 ml	2.00	.020	.71
Ethyl benzene	3.9 ml	3.37	.032	1.14
Naphthalene	4.8 gm	4.80	.038	1.36
Biphenyl	4.8 gm	4.80	.031	1.11
Tetradecane	8.5 ml	6.48	.032	1.14
Hexadecane	8.3 ml	6.55	.028	1.00
Eicosane	4.8 gm	4.80	.017	.61

TABLE 2. COMPOUNDS SCREENED FOR INHIBITORY EFFECTS

Heptachlor (Hept)	o-Chlorophenol (o-Cl-P)
Naphthalene (Nap	Endrin (End)
Mirex (Mir)	Methoxychlor (Meth)
Biphenyl (Bip)	Pyrene (Pyr)
1-Chloronaphthalene (1-Cl-N)	Pentachlorophenol (PCP)
Polychlorinated biphenyl (PCB)	Anthracene (Anth)

saturated with selected recalcitrant molecules. The slick was maintained in a closed continuous flow system by slow stirring on a magnetic stirrer. Liquid from a reservoir of pesticide- or aromatic hydrocarbon-laden water was directed slowly through the system at a rate to maintain the surface slick and a constant volume of liquid in the experimental vessel. The surface film was sampled at varying intervals by using 1 cm wide x 6 cm strip of polycarbonate material (no-hole Nuclepore). These were extracted in screw cap tubes with 2 ml of petroleum ether. Hexadecane and pollutant molecules were quantitated by gas chromatographic analysis.

SECTION 6

RESULTS AND DISCUSSION

ENVIRONMENTAL STUDIES

Microbial Populations

The microbial populations in surface films of estuarine, coastal, and pelagic waters were found to be higher than those previously reported (Table 3). Selected isolations for varied physiological types, in specific instances, revealed greater populations than indicated by nonselective medium for total aerobic heterotrophs. This finding further emphasizes the failure of a single nutrient medium to detect the total microbiota. The presence of such dense microbial populations suggests that the surface film is a site of significant metabolic activity. The varied numbers of physiological types in separate samples may reflect differential composition of films. None of the samples were collected from weathered hydrocarbon slicks or chronically oil-polluted areas. This fact may explain the relatively low number of hydro-carbonoclasts. A few samples from a fresh marine diesel spill (9-to 12-hr-old) yielded sparse numbers of microorganisms suggesting possible inhibition or dispersion of the normal film flora. The microbial populations of surface films may be influenced by the physical and chemical nature of the slick, the extent of the slick, the prevailing meteorological conditions, and the age of the slick.

Chemical

Analysis of extracts from large membranes indicated the presence of numerous electrophilic compounds not found in extraction of membranes without adsorbed surface film. Such compounds were observed most frequently in heavy slicks found in regions of intense biological activity or in regions subject to industrial output or effluents from sewage treatment facilities. Extracts of selected samples have been provided to the EPA Laboratory, Gulf Breeze, Florida, for more finite analytical studies.

LABORATORY STUDIES

Initial Studies

Characterization of the aerobic heterotrophs randomly isolated from Marine Agar 2216 illustrated that the majority of the isolates from coastal and estuarine films were proteolytic, amylolytic, and capable of growth on freshwater medium. None of these isolates utilized hydrocarbons or produced reactions on spirit blue medium. Studies of interference of growth of 41 isolates as determined by a modified antibiotic sensitivity test with discs

TABLE 3. CONCENTRATION OF MICROORGANISMS FROM SURFACE SLICKS*

Sampling	Number of total Heterotrophs	Number Hydro-carbono-clastic	Number Proteo-lytic	Number Amylolytic	Number Lipolytic	Reference
	1050 highest 60 mean					Sieburth 1965
		31 cm^2 highest				Gunkel 1973
	10^3 mean					Crow et al. 1975
	2.5×10^5 1×10^4	3×10^2 2.5×10^2	2×10^3 1×10			Crow et al. 1976
1	1.2×10^2	6	<1	23	--	Current data
2	8.1×10^2	2.7×10^2	<1	1.2×10^2	--	
3	5.3×10^4	2.7×10^3	2.9×10^3	3.5×10^4	--	
4	4.8×10^4	2.7×10^4	1.2×10^3	5.2×10^2	--	
5	2.3×10^3	43	1.2×10^2	6	--	
6	<1	<1	<1	<1	--	
7	35	<1	6	<1	--	
8	17	<1	<1	<1	--	
9	1.7×10^2	3	6	1.7×10^2	17	
10	57	--	<1	29	6	
11	57	4	<1	23	<1	
12	5.8×10^3	--	4.6×10^2	1.1×10^3	8.1×10^3	
13	5.8×10^3	25	5.2×10^2	3.9×10^3	--	
14	1.5×10^6	--	2.9×10^3	3.8×10^4	3.8×10^5	
15	1.15×10^6	--	1.7×10^4	2.9×10^5	6.9×10^4	
16	2.8×10^7	--	4.6×10^5	5.8×10^7	1.8×10^7	
17	<6	--	<6	<6	<6	
18	6.3×10^3	--	<6	8.1×10^2	5.2×10^2	
19	6.9×10^4	--	<6	6.9×10^2	4.6×10^2	
20	1.2×10^3	--	<6	8.0×10^2	35	
21	1.2×10^4	--	<6	9.8×10^2	1.1×10^3	
22	1.6×10^3	--	6	3.5×10^2	35	
23	<10	--	6	6	6.9×10^2	
24	1.2×10^2	--	6	6	<6	
25	1.8×10^2	--	6	60	69	
Mean	1.24×10^6	3.7×10^3	3.2×10^4	2.8×10^6	1.4×10^6	

*Number viable cells/cm^2

containing approximately 100 ppm heptachlor and methoxychlor showed no inhibition. Only one isolate demonstrated sensitivity to PCB formulations. Similarly, pesticides did not alter phosphatase activity. Concentrations of up to 100 ppm heptachlor and 200 ppm methoxychlor in basal medium fortified with 2 per cent ethanol did not alter (growth rates) of ethanol utilizing strains.

The growth of one yeast isolate, C. maltosa, representative of a strain associated with hydrocarbons, has given increased cell yields on hexadecane in the presence of heptachlor. Concomitantly, extractable levels of heptachlor in the culture broths have decreased. Results of a representative experiment are presented in Table 4. Though cell yields were slightly greater with larger heptachlor concentrations (Table 5) much greater amounts of heptachlor were unextractable at the higher concentrations. It is not clear whether this loss of heptachlor is due to metabolic uptake or to physical binding of heptachlor.

TABLE 4. HEPTACHLOR RECOVERY FROM HEXADECANE GROWTH MEDIUM

Organism	Initial Conc.		Disappearance	
	Heptachlor (μg)	Hexadecane (ml)	Heptachlor (μg)	Hexadecane (ml)
Candida maltosa (R-42, Ahearn)	0.0	0.1	0.0	0.0302
	956.0	0.1	85.1 (91 per cent)	0.0331
	9560.0	0.1	1405.3 (85 per cent)	0.0308

TABLE 5. DRY WEIGHT

Organism	Heptachlor Conc. (μg/ml hexadecane)	Dry Weight (μg/ml x 10^{-4})
Candida maltosa (R-42, Ahearn)	0.0	7.70
	956.0	7.52
	9560.0	9.19

Eight days after adding 6.748 x 10^5 CPM of labeled heptachlor to the culture system, 6.734 x 10^5 CPM, (99.79 per cent) could be recovered from

the various fractions (see Table 6). The amount of $^{14}CO_2$ trapped represented only 0.385 per cent of the total activity added, while the total recovery from the system was 100.178 per cent.

TABLE 6. RECOVERY OF ^{14}C LABEL FROM HEPTACHLOR FOLLOWING EIGHT DAYS OF INCUBATION WITH C. MALTOSA

Item	CMP	Per cent Recovery
Counts added	6.748×10^5	---
Counts recovered from culture	6.734×10^5	99.792
Counts recovered as CO_2	0.026×10^5	0.385
Counts recovered, total	6.760×10^5	100.178

The oil fraction (hexadecane or metabolites of hexadecane) contained the highest per cent of the label (82 per cent). The supernatant, aqueous fraction cells, and oil fraction cells contained from 2.6 to 8.5 per cent of the total label added (see Table 7), while the remaining fractions contained less than 1.0 per cent of the total ^{14}C added. It is of interest to note that even though the oil fraction contained the greatest isotope activity, the cells recovered from the aqueous fraction contained 53 per cent more radioactivity than those from the oil fraction. This for entirely in terms of numbers of cells in each fraction, for when calculated on the basis of CPM/ml of cells, the aqueous fraction cells still contained 9 per cent more activity than the oil fraction cells.

TABLE 7. RECOVERY OF ^{14}C LABEL FROM VARIOUS FRACTIONS OF YEAST CULTURE AFTER EIGHT DAYS INCUBATION WITH ^{14}C-HEPTACHLOR

Item	CPM	Per cent Recovery
Total counts added	6.748×10^5	---
Counts recovered	5.543×10^5	---
Oil fraction	5.543×10^5	82.143
Cells	0.576×10^5	8.536
Supernatent	0.377×10^5	5.587
Oil fraction cells	0.178×10^5	2.638
Rinse	0.018×10^5	0.267
Culture flask rinse	0.042×10^5	0.622
Total	6.734×10^5	99.793

The PE extraction of these cells revealed that only 42.0 per cent of the label remaining within the cells could be removed with PE. When one compares this with the 98.5 per cent recovery of ^{14}C-heptachlor from distilled water using PE, it becomes evident that (1) the heptachlor is not free to be extracted due to some form of binding, or (2) that the labeled compound is no longer heptachlor but one of its less soluble metabolites.

The data indicate that the majority of the ^{14}C-heptachlor remained solubilized in the hexadecane. However, some was found in all of the fractions that resulted from centrifuging the yeast containing media. Moreover, the loss of label due to metabolism to $^{14}CO_2$ appeared to be negligible; as did loss due to glass binding. This does not mean, however, that certain fragments of the molecule were not being utilized for energy production by the organisms, only that the portion of the molecule containing the label was not being utilized for CO_2 production. In fact, the extraction of the cells, with various solvents has given preliminary evidence that the heptachlor is being broken down, leaving a metabolite of metabolites which are not as soluble in PE as the heptachlor. The radioactivity remaining in the cellular fractions could have been due to: (a) hexadecane containing ^{14}C-heptachlor adhering to the outside of the cell, (b) the hexadecane containing ^{14}C-heptachlor located within the cells in vacuoles, (c) the ^{14}C-heptachlor being degraded and the ^{14}C incorporated into cellular structure, or (d) metabolites of hexadecane bound to or within the cell. The radioactivity remaining within the cells may be due to a combination of all these factors; however, the importance of each factor has not yet been determined.

In studies of the utilization of synthetic crude by C. lipolytica and C. maltosa, heptachlor consistently increased the utilization of synthetic crude over systems with synthetic crude alone (Table 8). With the addition of glucose and glycerol this effect was not evident. The recoverable amounts of naphthalene and biphenyl from the synthetic crude were consistently reduced after growth of both yeasts (Table 9). Neither of these compounds supported the growth of the yeasts as sole sources of carbon. Examination of molar utilization ratios of various components of synthetic crude indicate that the per cent reduction in the recovery of naphthalene and biphenyl is always greater in the presence of heptachlor (Table 10). This suggests that heptachlor is preferrentially affecting the uptake of naphthalene and biphenyl in these systems.

Studies of Selected Metabolic Types

Fifty-three selected isolates from the four major physiological groups of bacteria from surface slicks showed variable responses to chlorinated and aromatic hydrocarbons (Table 11). PCP, 1-chloro-naphthalene, and o-chlorophenyl were the most inhibitory compounds, whereas only a few strains were inhibited by heptachlor, methoxychlor, endrin, and mirex. The hydrocarbonoclastic group proved to be suprisingly sensitive to naphthalene, biphenyl, PCB 1016, and 1-chloro-naphthalene when grown in a peptone-based media, whereas the aerobic heterotrophs were less affected. The inhibition or stimulation of growth by various compounds appeared dependent upon the medium. The preceeding results (Table 11) were determined with a conventional nutrient medium. In a basal salt medium with hexadecane as the carbon

TABLE 8. UTILIZATION OF SYNTHETIC CRUDE OIL BY *C. LIPOLYTICA* AND *C. MALTOSA*

Item	*C. maltosa*	*C. lipolytica*
Synthetic crude	35.65*	26.27
+ Glycerol	25.20	29.20
+ Glucose	26.77	23.85
+ Methoxychlor	30.85	30.70
+ Heptachlor	47.10	47.20

*Per cent utilization based on uninoculated control
Each value is the result of average of duplicate flasks for two separate experiments

TABLE 9. UTILIZATION OF SYNTHETIC CRUDE COMPONENTS BY *C. LIPOLYTICA* AND *C. MALTOSA*

	Ethyl-benzene	Naphtha-lene	Biphenyl	Tetra-decane	Hexa-decane	Eico-sane
C. maltosa						
Synthetic Crude	29.3*	23.5	17.3	43.1	44.1	43.8
+ Glycerol	17.5	17.6	21.8	31.3	30.7	30.2
+ Glucose	26.7	20.3	23.1	26.2	28.2	27.6
+ Methoxychlor	22.5	23.6	29.9	36.3	36.8	37.2
+ Heptachlor	49.6	50.4	52.6	52.9	52.6	50.3
C. lipolytica						
Synthetic Crude	29.4	14.6	34.6	32.9	32.7	35.3
+ Glycerol	27.8	20.0	19.6	36.8	36.1	29.8
+ Glucose	46.7	22.0	21.8	23.6	22.7	23.8
+ Methoxychlor	14.4	26.4	29.2	32.7	35.5	26.6
+ Heptachlor	49.6	44.8	47.5	49.0	47.9	41.0

*Per cent utilization compared to an uninoculated control

TABLE 10. MOLE UTILIZATION RATIOS OF SYNTHETIC CRUDE OIL COMPONENTS

	C. maltosa					
	Nap/ Hex	Nap/ Hex + Tetra	Nap/ Hex + Tetra + Eic	Bip/ Hex	Bip/ Hex + Tetra	Bip/ Hex + Tetra + Eic
Synthetic crude	.76	.36	.28	.81	.39	.30
Synthetic crude + Heptachlor	1.38	.65	.51	1.10	.51	.41
	C. lipolytica					
	Nap/ Hex	Nap/ Hex + Tetra	Nap/ Hex + Tetra + Eic	Bip/ Hex	Bip/ Hex + Tetra	Bip/ Hex + Tetra + Eic
Synthetic crude	.56	.25	.20	1.20	.55	.44
Synthetic crude + Heptachlor	1.31	1.00	.49	1.15	.88	.42

TABLE 11. EFFECT OF CHLORINATED AND AROMATIC HYDROCARBONS ON GROWTH OF SELECTED MICROORGANISMS

Physiological Type	Number Isolate Tested	COMPOUNDS (100 μg)											Total Inhibitory Responses
		O-Cl-P	I-Cl-N	1016 PCB	Nap	Hept	Meth	Bip	End	PCP	Mir	Pyr	
Aerobic Heterotroph	25	12/25	18/25	6/25	2/25	1/25	1/25	1/25	2/25	25/25	1/25	0/25	69/275
Lipolytic	9	5/9	7/9	0/9	4/9	0/9	0/9	4/9	0/9	9/9	0/9	0/9	29/99
Hydrocarbonoclastic	11 & 15	0/15	12/15	10/15	7/11	0/11	0/15	6/11	0/11	15/15	0/15	0/15	50/149
Amylolytic	4	3/4	4/4	1/4	3/4	1/4	0/4	2/4	4/4	4/4	0/4	0/4	16/44
Total	53.49	20/53	41/53	17/53	16/49	2/49	1/53	13/49	2/49	53/53	1/53	0/53	---

source, the effect of various compounds appeared organism-related (Table 12). In general, bacteria which showed the greater utilization of hexadecane were inhibited by a wider range of compounds. This phenomenon may be related to transport and concentration of the pesticide within the cell. The eleven cultures of C. lipolytica grown on a carbohydrate medium were more resistant to most compounds than the hydrocarbonoclastic bacteria (Table 13). In contrast to the bacteria, the yeasts were resistant to PCB 1016, naphthalene, and biphenyl. Both bacteria and yeasts were sensitive to 1-chloronaphthalene. In a hexadecane-based medium the yeast appeared to be less affected by addition of aromatic and chlorinated compounds than by bacteria (Table 14). In model surface slick systems, hexadecane films rapidly accumulated both naphthalene and PCP from subsurface water. PCP reached concentrations of .02 to .03 per cent in hexadecane after 2 days of continuous exposure to water containing less than 80 ppm pentachlorophenol. Concentrations of .08 per cent were measured after an additional 24 hr. Recalcitrant molecules in experimental accumulation studies rapidly reached concentrations several orders greater than the magnitude that inhibited representative surface slick isolates. Accumulation or sequestering of recalcitrant molecules within a surface film may alter essential metabolic processes with this system.

TABLE 12. EFFECT OF AROMATIC AND CHLORINATED HYDROCARBONS (10 PPM) ON HEXADECANE METABOLISM BY SURFACE SLICK BACTERIA

Culture No.	Hexa-decane control	O-Cl-P	1-Cl-N	PCB 1016	Nap	Hept	Meth	Bip	End	PCP	Mir	Pyr	Total Inhibitory Responses
19-2	34[a]	0	+++	0	0	0[b]	0	+++	0	0	+++	+++	4/11
18-3	59	++	0	++	0	+	-	++	++	+++	0	0	6/11
9-1	50	0	+	0	0	+++	-	0	0	+++	-	+++	4/11
H-17	7	+++	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	10/11
H-16	84	0	-	0	-	+	0	0	-	0	-	-	1/11
H-19	52	0	0	+++	-	0	-	+	0	+++	-	0	3/11
H-14	72	0	-	+++	-	-	-	-	-	+++	0	-	2/11
H-28	35	+	0	-	0	-	+++	-	-	+++	+	+++	5/11
Total		3/8	2/8	4/8	1/8	4/8	2/8	4/8	2/8	6/8	3/8	4/8	

[a]per cent recovery hexadecane

[b]+ 10-20 per cent inhibition
++ 21-30 per cent inhibition
+++ greater than 31 per cent inhibition
- enhanced utilization
0 no effect

TABLE 13. INHIBITION OF C. LIPOLYTICA ISOLATE BY VARIOUS COMPOUNDS

No. Tested	100 µg											Total Inhibitory Responses
	O-Cl-P	1-Cl-N	PCB 1016	Naph	Hept	Meth	Bip	End	PCP	Mir	Pyr	
11	4/11	11/11	3/11	0/11	0/11	0/11	0/11	0/11	11/11	2/11	3/11	34/121

TABLE 14. EFFECT OF AROMATIC AND CHLORINATED HYDROCARBONS AT 10 PPM ON HEXADECANE METABOLISM BY *C. LIPOLYTICA* MUTANTS

Mutant No.	Hexa-decane control	O-Cl-P	1-Cl-N	PCB 1016	Nap	Hept	Meth	Bip	End	PCP	Mir	Pyr	Total Inhibitory Responses
37-1	67[a]	-	0	-	0	0[b]	0	0	+++	0	0	0	1/11
5-2	96	0	0	0	0	0	0	0	0	0	0	0	0/11
12-1	28	+++	0	+++	+++	++	+	+++	0	+++	0	0	7/11
5-1	27	+	0	+	+++	0	0	++	0	+++	0	0	5/11
8-2	28	0	0	0	+	0	++	++	+	++	0	0	5/11
4-2	41	0	0	0	0	0	0	-	0	+	0	+	2/11
25-2	83	0	0	0	0	0	0	0	0	-	0	0	0/11
Total		2/7	0/7	2/7	3/7	1/7	2/7	3/7	2/7	4/7	0/7	1/7	

[a]Per cent recovery hexadecane

[b]+ 10-20 per cent inhibition
++ 20-30 per cent inhibition
+++ greater than 30 per cent inhibition
- enhanced utilization
0 no effect

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16. ABSTRACT Estuarine surface films from Escambia Bay, Florida, and adjacent waters were sampled by using the membrane adsorption technique to enumerate microbial populations. Samples of the upper 10 μm of estuarine surface films yielded microbial populations up to 10^8 ml^{-1} or 10^5 cm^{-2}. These populations were 10 to 100 times greater than those in underlying waters of 10 cm. Predominant bacteria in surface films as isolated on Marine Agar were motile, nonpigmented, gram-negative rods. Colony-forming units of yeasts and molds on Mycological Agar prepared with 50 per cent seawater were found in concentrations to 10^4 ml^{-1} or 28 cm^{-2}. The predominant surface film isolates from Marine agar were proteolytic and amylolytic but exhibited only weak to negligible hydrocarbonoclastic and lipolytic activities. A greater proportion of the surface-film bacteria, as compared to those at 10 cm depth, were capable of growth on fresh-water media. With selective isolation media, amylolytic, and lipolytic bacteria appeared to comprise a more significant proportion of the total population. Twenty-one representative bacteria, yeasts, and filamentous fungi from initial sampling of surface microlayers were tested for the effects of selected pesticides on utilization of various substrates. One bacterium was sensitive to PCB formulations. In subsequent studies with 53 isolates representative of more diverse physiological groups, o-chloronaphthalene, PCB 1016, and pentachlorophenol were inhibitory to a large portion of the isolates and heptachlor, biphenyl, pyrene, and PCB 1016 significantly reduced hexadecane utilization.

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