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Ecological Research Series

Impact of the Use of Microorganisms on the Aquatic Environment

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IMPACT OF THE USE OF MICROORGANISMS ON THE AQUATIC ENVIRONMENT

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ABSTRACT

This report contains the proceedings of a symposium-workshop sponsored by the EPA Gulf Breeze Environmental Research Laboratory to determine the possible impact of artificially introducing microbial insect control agents or oil-degrading agents into the aquatic environment. The efficacy and safety testing, especially against non-target aquatic organisms, for use of bacteria, viruses, fungi, and protozoa to control aquatic insect pests is discussed with remarks of panel members representing government, academia, and industry. Special attention is given to persistence of pathogens in aquatic environments as well as control of aquatic weeds and other non-insect pests.

The use of microorganisms to clean up oil spills in aquatic environments is discussed by industrial, academic, and governmental scientists. Special considerations are given to selection of hydrocarbonoclastic microorganisms and use of these microorganisms in special environments--Arctic regions and Louisiana salt marshes.

Summary papers are presented for each panel concerned with microbial pesticides and one summary for the session on microbial degradation of oil.

This symposium-workshop was held in Pensacola Beach, Florida, in April, 1974.

ACKNOWLEDGMENTS

This symposium-workshop was hosted by the EPA, Gulf Breeze Environmental Research Laboratory, Gulf Breeze, Florida. The cooperation of the GBERL personnel was greatly appreciated, especially the efforts and encouragement of GBERL's Director, Dr. T. W. Duke. Principal contributors to the preparation and operation of the symposium were Ms. Chiara Shanika, Ms. Lynda Keifer, Mr. Scott Cassidy, and Ms. Gerta Guernsey, GBERL, and Ms. Vicki Tayoe, Ms. Glennis Mitchell, Mr. Norm Smith, and Dr. Warren Cook, Georgia State University.

The exceptional efforts of the session chairmen, Dr. Donald G. Ahearn, Georgia State University, and Dr. Carlo M. Ignoffo, U.S. Department of Agriculture, are most gratefully acknowledged. Special thanks go to all the participants whose remarks helped make the symposium a success.

I wish to acknowledge the rapid and excellent work of Ms. Mary H. Alston, Baton Rouge, Louisiana, in transcribing, editing, and typing the many tapes and manuscripts for this volume. The untiring and generous efforts of the co-editors, Dr. Donald G. Ahearn, and Dr. Samuel P. Meyers, Louisiana State University, are most gratefully acknowledged.

Al W. Bourquin
Program Chairman

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INTRODUCTORY REMARKS

Al W. Bourquin, Program Chairman

Welcome to Gulf Breeze and the symposium-workshop "Impact of the Use of Microorganisms on the Aquatic Environment." The purpose of this symposium is to assess the impact of biological control agents on the aquatic environment. We have divided the symposium into two sessions, the first dealing with biological control of insects and other aquatic pests and the second session considering the impact of introducing hydrocarbon-degrading microorganisms into the aquatic environment.

The objectives of this workshop are: (1) to determine the status of current research on the environmental impact of artificially introduced microorganisms, (2) to assess future areas of investigation into the environmental impact of artificially introduced microorganisms, and (3) to promote communication and exchange of information. The workshop will include short addresses followed by panel discussions. Dr. Carlo M. Ignoffo, chairman of Session I, and Dr. Donald G. Ahearn, chairman of Session II, will present a synopsis of the panel discussions on the final day with recommended guidelines for future research.

WELCOMING REMARKS

Thomas W. Duke, Director
EPA Gulf Breeze Environmental Research Laboratory

It is my pleasure to welcome you to the Gulf Breeze Environmental Research Laboratory and to tell you that we look forward to having you tour our facilities tomorrow afternoon.

I would like to take a few minutes to tell you briefly of our research activities at the Gulf Breeze Laboratory and how we became interested in this workshop. We work in a rather emotional field in that we are developing a scientific data base on the effects of chemicals and natural organics on marine organisms. This includes, of course, pesticides. I admit to you that our data base falls far short of what we would like it to be. The development of new chemicals and new uses of old ones have outstripped our capacity to develop data on their effects.

Recently, we have seen new interest developing on the use of biological control agents to control agricultural pests and to degrade hydrocarbons. Thus we have become more interested in the impact of these uses of microorganisms on the aquatic environment. Personally, I am well pleased to see this movement toward biological control. It pleases

me to consider using the forces of nature to control pests and I think that the results could be a cleaner environment than we enjoy at the present time. At the same time, I see a need, based on past experience, of having information on the impact of these control agents on non-target portions of the aquatic environment.

Interest in this impact was recently stimulated by research conducted by Dr. John Couch of our laboratory. He was asked to make some pathological examinations of pink shrimp that were used as test animals in a study on the effect of Aroclor 1254, a polychlorinated biphenyl, on certain estuarine organisms. He discovered some large triangular crystalline inclusion bodies in the hepatopancreas of the shrimp. Through electron microscopy he found that these inclusion bodies contained rod-shaped virus particles and that these virus particles are similar to those of a nuclear polyhedrosis virus. What does this occurrence mean? We do not know at present since we have not yet made a proper evaluation. Also, we do not know what relation the chemical has to manifestation of this virus.

Dr. Bourquin mentioned that the purpose of this meeting was to provide a forum for scientific discussion of the impact of the use of microorganisms on the aquatic environment. As we listen to the presentations and the discussions, I am sure that many questions will arise. For example, what pathogens are under consideration now and will be in the future? What kinds of field testing have been accomplished with which organisms? How specific are these pathogens? Have tests been conducted to evaluate the safety of organisms other than the target pest?

On Thursday morning, in the preparation of the summary, I ask that chairmen of the different sessions address these questions in their summaries.

I

ENVIRONMENTAL CONSIDERATIONS ON MICROBIAL PESTICIDES

USE OF BACTERIA FOR CONTROL OF AQUATIC INSECT PESTS

Samuel Singer*

INTRODUCTION

The choice of bacterial candidates for control of aquatic insect pests remain the bacilli. By now you are all aware of the successes accorded *Bacillus thuringiensis* against agricultural crop insect pests in a terrestrial environment. For the record of *B. thuringiensis* one need only refer to the rash of publications and recent conferences: the remarks of Bourgerjon at the Atlanta conference on the "Safety of Biological Agents for Arthropod Control" in Atlanta, last spring; the Fifth International Colloquium on Insect Pathology and Microbial Control at Oxford this past fall; the National Academy of Sciences report "Mosquito Control: Some Perspectives for Developing Countries" (2); a symposium on Microbial Insecticides held last summer at the Society of Industrial Microbiologists meeting (4). It would be more profitable to discuss the newer bacilli candidates, the most outstanding example of which is *Bacillus sphaericus*/SSII-1. This strain, according to the calculations of Goldberg (11), will probably require as little as 56.7 g of dry material per acre of application. This should make this bacterial control agent truly competitive with chemicals.

The work discussed here includes input from several people. It has been perhaps our naive assumption that the best source for potential bacterial candidates against specific insects or invertebrate targets would be from natural epizootics of these invertebrates. To date we have been successful in isolating *B. thuringiensis* strains active against the Indian-meal moth (*Plodia interpunctella*) from *B. thuringiensis* infected Indian-meal moths. Similarly we have been successful in isolating bacilli active against *Culex* from *Culex* larvae infected with these bacilli. Given time we feel we could do the same for *Aedes*, *Anopheles*, biting flies and schistosome-bearing snails.

When we propose a new biological control agent factors of efficacy, safety, environmental impact and economic applicability must be considered (21). In terms of microorganisms, efficacy includes isolation, identification and biological activity; safety includes human, mammalian and phytological toxicity; environmental impact equates with impact on target and non-target invertebrates as well as microbiological/ecological considerations; while economic applicability denotes further development in terms of economic, commercial and socio-political considera-

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tions. Let us consider each of these four aspects of a new bacterial insecticide, particularly in terms of the definitions expressed.

EFFICACY

Until recently, the choice of bacterial candidates for the control of aquatic insect pests has been primarily strains of *Bacillus thuringiensis* such as BA068. Reeves and Garcia (19, 20), using BA068, reported its successful use under laboratory conditions against three species of *Aedes*, at a level of 10^6 spores/ml. We have tested BA068 against our laboratory culture of *Culex pipiens* var. *quinquefasciatus* and found it to be inactive against these larvae. However, we did find several type varieties of *B. thuringiensis* active against our *Culex* larvae at a level of 10^7 cells/ml. Goldberg (personal communication) noted that *B. thuringiensis* strain HD-1 (the commercial *B. thuringiensis* strain) as well as its crystal, is just as active as BA068 against several *Aedes* species, but not against *Culex*. Several groups, including one commercial group, presently are investigating use of these and other *B. thuringiensis* isolates for control of mosquito larvae,

Recently, we isolated two new groups of bacilli, with activity against mosquito larvae, from World Health Organization (WHO) material (22) (Table 1). This material originated in Delhi, India, from field samples of dead mosquito larvae that succumbed to natural epizootics caused by the bacilli. In the past few years similar field material has been sent to the World Health Organization International Reference Center (WHO/IRC) for diagnosis of Diseases of Vectors (under the direction of Dr. John D. Briggs) for preliminary diagnosis. These accessions showing *Bacillus* infections have been forwarded to us for further development. Table 2 lists many of the accessions that are available and in process of further investigation. Note that the material originates from countries forming a geographical arc from Korea in the western Pacific to Zambia in East Africa and Nigeria in West Africa.

The WHO/IRC material which forms the basis of our recent work originated in Delhi, India. One of these new bacterial candidates, *B. sphaericus*/SSII-1 (morphological group III bacilli, Table 3) has been found to be 10,000 X more active than previously examined strains of bacilli; as few as 10^2-10^3 cells/ml result in LD_{50} and LD_{90} effects against *C. pipiens* and *C. tarsalis*. A second group of bacilli (morphological group II bacilli) form a complex of strains belonging to the *B. alvei-B. circulans-B. brevis* group and also have shown larvicidal activity but not to the same degree.

According to modern microbial physiology all of the bacterial insecticidal "toxins" that have been found to date have the characteristics of secondary metabolites (23, 24). All are generated during early prespore stages of sporulation (after the cells have stopped dividing) and all possess no obvious function in cell growth. The *B. thuringiensis* crystal is a stable material from a cellular point of view. It persists and is released at the time that the spore is released. The activity

of the sphaericus toxin on the other hand, while formed during stages of secondary metabolism, appears to peak in activity prior to mature spore formation, with an eventual decay of one or two logs of activity. The heat labile soluble toxin produced by the group II bacilli also is generated during secondary metabolism, but does not usually appear to decay once the spore is formed. These secondary metabolic aspects bear heavily on the practical considerations involved in eventual field application as well as in consideration of initial efficacy.

Table 1. COMPARISON OF INSECTICIDAL ACTIVITY OF SEVERAL ISOLATES FROM DELHI, INDIA, ACCESSIONS #1321 (I-XV) WITH ORIGINAL KELLEN STRAIN

Isolate	LD_{50}*
*B. sphaericus***/II-1	2.3×10^2 cells/ml
B. sphaericus/IV 8-B	2.4×10^3 cells/ml
B. alvei(aberrant)/III-3	1×10^6 cells/ml
B. circulans/alvei intermediate/VI-12	1×10^6 cells/ml
B. alvei/VII-6	1×10^6 cells/ml
Original Kellen strain *B. sphaericus*/K	1×10^7 cells/ml

*LD_{50} = number of viable cells in population of original broth culture needed to kill 50% of test insect, *Culex pipiens* var. *quinquefasciatus*.

**All *B. sphaericus* cultures listed can be considered related to var. *fusiformis*.

The pathology of SSII-1 against *Culex* has been carried out by Dr. Elizabeth Davidson of Arizona State University (6). Briefly (Table 4), there does not appear to be any gross tissue damage to the larvae at cell concentrations above the LD_{50} level. No bacterial cells have been found outside of the peritrophic membrane. Supernatants of the final whole cultures of SSII-1 show no insecticidal activity, indicating that the "toxin" is probably associated with the cell itself. Evidence points to the action of a toxin. Examining gut populations of treated cells she has found populations of SSII-1 of the order of 10^5 cells/larva with no apparent increase in number with time. In fact there is a decrease in numbers if anything. When bacterial populations were treated with chloroform only a one log drop in insecticidal activity occurred even while the detectable viable cell count was reduced to zero. This chloroform treatment opens the possibility of using

chloroform-killed cells as a "chemical" insecticide. We do not know what the toxin sensitive site in the insect is, nor how death occurs. We do know that death can come as quickly as 12 hr.

Table 2. WHO/IRC ACCESSIONS AND THEIR SOURCE

	Vector material	No. of Accessions	Bacillus, Group Morphological Group
Korea	*Culex fatigans*	1	(Bacillus)*
	Vermiculite	1	(Bacillus)
The Philippines	*Culex pipiens quinquefasciatus*	1	(Bacillus)
Indonesia	*Culex pipiens quinquefasciatus*	1	(I & II)
Australia	*Culex annulirostria*	1	(II)
Thailand	*Culex pipiens quinquefasciatus*	2	(II)
Burma	*Culex fatigans*	2	(II)
India	*Aedes aegypti*	16	II & III, (II & III)
	Culex fatigans	5	Bacillus), (II)
Zambia	Cattle hair scrapings and ticks	4	(Bacillus)
Nigeria	Ecological samples:		
	Anopheles spp.	14	*B. sphaericus*/II-1
	Culex spp.		(Bacillus)
California	*Aedes dorsalis*	2	*B. thuringiensis*

*Parentheses denote presumptive.

Table 3. EFFICACY OF *Bacillus sphaericus*/SSII-1: MICROBIOLOGY

Origin: WHO/IRC Accession #1321/II Delhi, India

Classification: *Bacillus sphaericus* var. *fusiformis*

Biogenesis of biologically active component:

Associated with cell itself (heat labile).

No activity in supernatant of Final Whole Cultures.

Appears during secondary stage of metabolism.

Chloroform treatment of populations results in one log drop in activity.

Most active when grown in synthetic medium.

Two logs less activity when grown in non-synthetic medium.

Active when grown in "fresh frozen" alfalfa infusion.

Table 4. EFFICACY OF *Bacillus sphaericus*/SSII-1: BIOLOGICAL ACTIVITY

Biological Activity (LD_{50})*	
Culex pipiens var. *quinquefasciatus*	10^2-10^3 cells/ml
Culex tarsalis	10^2-10^3 cells/ml
Aedes aegypti	10^6 cells/ml
Anopheles spp.	10^6 cells/ml
House fly	Inactive
Plodia interpunctella	Inactive
Lasioderma serricorne	Inactive
Australorbis glabratus	Partially active

Histopathology

No apparent gross tissue damage.

No *Bacillus* found outside of peritrophic membrane.

Gut population 10^5 cells/larva, no further increase, usually a decrease of gut population.

Death as early as 12 hours.

Most activity apparent by 2 days.

For low population numbers, activity continues for 7 days.

With low spore preparation, instars I-IV equally sensitive.

*LD_{50} = Number of cells/ml of bacterial population killing 50% of test animals.

Of the cultures that we have been working with, *B. sphaericus*/SSII-1 is also closest to field trial. Last summer this strain was tried at the Desplaines Mosquito Abatement District in Lyons, Illinois, as well as in Kaduna, Nigeria. It was also tested by Leonard Goldberg of the University of California, Berkeley Naval Biomedical Research Laboratories. Goldberg found SSII-1 to be effective in his laboratory against *Culex pipiens* as well as against *Culex tarsalis* (11), at levels comparable to those reported previously (22). Dr. Calvin Alvarez of the Desplaines Mosquito Abatement District tried SSII-1 against field-collected *Culex pipiens* var. *pipiens* and found LD_{50} values in the order of 10^3-10^4 cells/ml. In the Kaduna test, SSII-1 was active against *Anopheles* in the order of 10^6 cells/ml. Our own most recent experience

shows SSII-1 to be active against *Aedes aegypti* in the order of 10^6 cells/ml. SSII-1 is as active as our best *B. thuringiensis* strains against *Aedes* and *Anopheles* and at least 10,000 X more active against *Culex* species.

To illustrate the rate at which *B. sphaericus* work has been going recently, I have just recently learned from Goldberg that he has isolated substrains of SSII-1 capable of high percent sporulation while still retaining high insecticidal activity. In terms of efficacy and depending on available funding, we hope to examine our isolates from WHO/IRC accessions in order to generate potential field candidates against *Aedes*, *Anopheles*, biting flies and schistosome-bearing snails.

SAFETY

Both national and international programs have been developed to assay, monitor and evaluate the impact of chemical insecticides. The World Health Organization has adopted a seven-stage evaluation of chemical insecticides necessary for a chemical product to be adopted for use by WHO (1). No comparable program exists in the international area for biological (microbiological) agents although one is being prepared. At the national level strict federal regulations exist for chemical and guidelines for new viral agents (5). However, no guidelines exist for safety testing or environmental impact of bacterial control agents (7), although *B. thuringiensis* has been registered for many food crops. Safety testing of microbial agents, whether their ultimate fate is for a terrestrial or an aquatic environment, would obviously be the same. Basically, it will have to be shown that the insect pathogen cannot act as a pathogen for man, other vertebrates or plants. The differences between use in a terrestrial or an aquatic environment will bear on its impact on the normal, non-target, invertebrate and plant taxa, at each particular trophic level or niche of the particular environment. The basic difference will be in terms of its environmental impact, rather than safety to man and his economically important plants and animals. For instance, *B. thuringiensis* has been used mainly in terrestrial environments. Safety information gathered should be equally valid if *B. thuringiensis* were to be used in an aquatic habitat. Information is needed on its impact on the plant and animal taxa of the aquatic environment where it will be used. Unlike a new class of chemical insecticide, where generally little or nothing is known of its toxicity, a new class of microbial insecticides usually concerns a known species. Thus, there is often some indication of its potential danger, at least its potential virulence.

The consequence of renewed interest in bacterial agents, as well as the emergence of new groups of entomogenous bacilli with unsuspected entomophilic properties, hastens the need for acquiring information concerning both safety and impact of these agents on the aquatic environment. However, little information is available in terms of these newer bacterial candidates, but what data are available are encouraging.

The morphological group II and III bacilli, when compared with other bacilli, are relatively inert metabolically and physiologically. These groups of bacilli are also very poorly understood from a taxonomic sense. In terms of specificity, we have found that with *B. sphaericus*/SSII-1, *Culex* is most sensitive, requiring in the order of 10^2-10^3 cells/ml for an LD_{50}-LD_{90} effect. In contrast, *Aedes* and *Anopheles* are less sensitive, requiring on the order of 10^6 cells/ml for LD_{50} effects. Recently we have found that the Indian-meal moth (*Plodia interpunctella*), the cigarette beetle (*Lasioderma serricorne*) and the house fly appear to be insensitive to this strain. We find some indication that the schistosome-bearing snail, *Australorbis glabratus*, may be partially sensitive.

According to Gordon et al. (12), with the exception of the Anthrax bacillus, members of the genus *Bacillus* are generally considered to be non-pathogenic for man. A strain of *B. sphaericus*, however, was reported by Farrar (8) as the causal agent of a fatal disseminated infection, but close examination of this report shows that the "infected" individual was in a highly traumatized state. This is a case where specificity becomes important. There are as many strains of *B. sphaericus* which are inactive against mosquito larvae as there are active ones. Similarly, assuming Farrar's correct identification of the causative agent in his patient, this does not necessarily preclude the use of *B. sphaericus* against aquatic insect pests, especially if adequate and careful safety tests are done on prospective field test material.

We have not as yet done any extensive mammalian toxicity studies of our new bacterial candidates. This requires a carefully thought-out program by workers in several disciplines, which at the moment is beyond our means. However, these studies are under consideration and will be instituted especially if new field trials are to be developed under WHO auspices.

ENVIRONMENTAL IMPACT

In the area of environmental impact, we have planned: 1) study of the effect of these bacilli against isolated insect and invertebrate target and non-target systems; 2) study of the fate of populations of these bacilli in simulated and natural aquatic environments.

IMPACT ON ISOLATED INSECT AND INVERTEBRATE TARGET SYSTEMS

Two areas of impact become important when one considers the invertebrate taxa. These are: a) the effect of these new agents on invertebrates that are predators or are able to parasitize these vector and pest targets; b) the effect of these new agents on invertebrates that are non-vector, harmless, but important members of the aquatic ecosystem. One of the benefits of biological control is specificity, which translates to mean that the natural predators and parasites are allowed to contribute to the total control of the aquatic pest(s). It is therefore appropriate to include in our examination the effect of these

control agents on several invertebrates that may eventually become members of a biological control complex.

Table 5 lists non-pest and pest organisms selected as representatives of their respective taxa in fresh-water systems. Three systems in particular are noted.

Table 5. SELECT TARGET AND NON-TARGET AQUATIC ORGANISMS

Taxa	Test Group
I. Parasites and Predators of Mosquito Larvae:	
Protozoa	*Tetrahymena*
Coelenterata	*Hydra*
Platyhelmenthes	*Dugesia* (Planaria)
Mollusca	*Limnaea*
Annelida	Fresh water Oligochaetes
Arthropoda	
Crustacea	*Daphnia*, *Gammarus*, *Cyclops*, crayfish
Insecta	
Odonata	Damselfly and dragonfly numphs
Ephemoroptera	Mayfly larvae
Diptera	*Toxorynchites*
Chironemidae	Chironomid midges
II. Others:	
Hymenoptera	
Apidae	Honey bee
Diptera	
Simuliidae	Black flies

Toxorynchites are important potential biological control agents since they are effective predators of all mosquito larvae, even members of their own species. Transmission of disease from prey to predator was shown by Nolan et al. (18) with *Coelomomyces* fungus infection of *Toxorynchites* from its prey *Aedes*.

Honey bees may be expected to contact *B. sphaericus* while gathering water from treated areas, especially in areas such as the southwestern United States, where the supply of open water is limited and likely

includes mosquito habitats. Testing of microbial agents, principally *B. thuringiensis* against honey bees has been reviewed (3) and little evidence of pathogenicity of *B. thuringiensis* against the bees is noted. The effects of *B. sphaericus* against the honey bee should still be tested. Methods are available (15, 16, 17) that have proven satisfactory in testing herbicides against bees. An agreement has been reached with USDA-ARS bee laboratory in Tucson, Arizona, for us to test *B. sphaericus* at their facility.

Among those aquatic organisms in North America classified as detrimental to man, the black fly ranks second only to the mosquito as a pest. Though black flies utilize bacteria as food (19), few bacterial pathogens have been reported (13, 14) and none has been extensively tested as a control agent. Therefore, we feel that preliminary testing of *B. sphaericus*, as well as other select isolates, against black fly larvae is worth pursuing.

FATE OF POPULATIONS OF BACILLI (*B. sphaericus*/SSII-1) IN SIMULATED AND NATURAL AQUATIC ENVIRONMENTS

Consideration of the impact of these bacterial candidates on invertebrate taxa, whether in isolated systems or in nature, is obviously important, yet insufficient to our needs. Since we are dealing with a two-component system, invertebrate and bacillus, the fate of the microorganism, both in the wild and in simulated ecosystems, bears a strong influence on the question of safety and environmental impact. Therefore, a well planned study of the fate of populations of the bacilli, both in a natural as well as simulated ecosystem, becomes important. With chemical there is the question of residue, with microorganism the question is one of survival of the physiologically active component(s), either the vegetative cell, the sporulating cell, the spore or some product of one of these. The strategy, of improving efficacy of application of microbial material, rests on the keystone of controlling a man-made epizootic. Control hopefully means a minimum of reapplication. It is ideally a feedback situation with the controlling agent persisting at some minimal level during stages of low numbers of target invertebates, and increasing sufficiently, due to "reinfection" of the target host, when the numbers of the target host itself increases. This ideal or theoretical ecological balance is yet to be demonstrated.

As noted earlier, the microbiological side of the two-component system, invertebrate-bacillus, has in the past been neglected. Survival of the *Bacillus* insecticide is comparable to residue of the chemical insecticide and bears heavily on the eventual environmental impact. Safety data always have been sought along with the effect of the insecticide on non-target invertebrates. But little has been done to determine the fate of the important factor--the microorganism itself. This does not diminish the importance of these other considerations.

Table 6 lists the approaches that we are interested in developing. The basic data to be gathered in each case are simply bacterial

population counts, both total viable and total spore counts. We are basically interested in what happens to high-spored and low-spored populations, and what happens to these insecticidal populations of SSII-1 over periods of time in the presence of "native water." By the latter is meant water from the various natural mosquito habitats. These could be marshland, pond (both intermittent as well as all year type), back water, and water from urban habitats such as drainage ditches and sewer lagoons. For "simulated standing systems" it would simply be observations of dosed native water in Pyrex dishes. For more active water systems, double spinner flasks are possible. The latter is a system of two flasks each magnetically stirred and separated by a semipermeable membrane. The wet-dry-wet system is projected as one wherein dosed native water is vacuum-dried, stored, and reconstituted with native water, all of which mimics a wet-dry-wet series of seasons. "Factors affecting population decline" implies study of the effect of bacterial flora, found in the native water, on the potential insecticide. The natural ecosystems series is almost self-explanatory. This is the non-simulated natural system whereby we a) simply follow the fate of the population of the insecticide in natural ponds dosed with the bacillus and b) examine the effect of the bacillus on invertebrates in the natural pond.

Table 6. THE FATE OF POPULATIONS OF *Bacillus sphaericus*/ SSII-1 IN SIMULATED AND NATURAL ECOSYSTEMS

I. In Simulated Ecosystems (Native Water*)
   A. Standing (Pyrex dishes)
   B. Moving (double spinner flasks)
   C. Wet-dry-wet sequence
   D. Factors affecting population decline
      1. Antibiosis plates
      2. Double spinner flasks

II. In Natural Ecosystems
   A. Fate of SSII-1 populations in natural ponds
   B. Effect of SSII-1 on target and non-target invertebrates in natural ponds.

*Native water = water from mosquito habitats.

At this moment ecological studies similar to those just described are being carried out as a result of the Kaduna experiments. We hope to interface with these studies, thereby providing information of trophic levels in similar ecological circumstances to provide baseline data for supporting ecological impact statements.

ECONOMIC APPLICABILITY

At this time, I would like to discuss mainly commercialization. In a recent letter from Abbott Laboratories I have the following figures. In the United States over 500,000 acres in agriculture are treated with *B. thuringiensis* and another 25,000-50,000 acres in forests. Total industrial production of *B. thuringiensis* exceeds one million pounds and worldwide use increases this figure to perhaps two million pounds. All manufactured products are produced primarily by two companies, Abbott and Sandoz, with Abbott the major portion, and a third company, Nutrilite, producing a minor component. Usage is expected to increase in 1974, especially since petrochemicals are in short supply. To quote Abbott's letter, "In summary, biological insecticides are not laboratory curiosities but viable alternatives to chemical control agents. They can be produced commercially in quantities equivalent to chemicals and fear of production inadequacies should not be a deterrent to use."

Two companies, Nutrilite and Abbott, have shown an interest in examining SSII-1. In a paper delivered before the California Mosquito Association Meetings this February, Goldberg (11) tells of plans to field test active spore preparations in Kerne County, California.

Data presented here deal with preparations of SSII-1 that are neat, that is, with none of the usual additives, baits, etc., generally found in final products of this nature. Thus, much work will have to be done in this regard.

SUMMARY

In dealing with impact of bacteria on aquatic environment I have not dealt extensively with safety in the sense of usual vertebrate toxicity. This area needs a full treatment in a symposium-workshop concerned with just this topic. It requires carefully thought out input from bacteriologists and insect pathologists as well as from mammalian pathologists, toxicologists, immunologists and others.

Guidelines for bacterial insecticides, whether aquatic or terrestrial, will have to be developed. In terms of safety guidelines for a new product, the manufacturer will have to bear the main burden of cost. The same safety tests outlined for the *B. thuringiensis* registration should play a role in development of these guidelines. One interesting paper with an excellent treatment on this aspect of toxicity, as well as initial field testing (10), reviews work with *B. moritai* as well as several *B. thuringiensis* strains.

The effect of adjuvants needed for the special aquatic situation also will undoubtedly influence these "guidelines." These problems will require careful thought and planning. What I would like to emphasize, however, are the problems concerning efficacy and environmental impact. We have been spoiled to some extent by the success of *B. thuringiensis* in that we have neglected other bacilli as well as other

bacterial genera. It has been too easy to stress development of very active fully sporulated materials. A major need is to develop "delivery" systems that will permit field use of non-sporulated material. In spite of the current feeling, one can more quickly harvest the fruits of laboratory efforts such as ours, so that the time lag between isolation and field use can be reduced. A time will come when an active material, such as *B. popilliae*, may not be able to be developed into a spored preparation.

In terms of environmental impact, decay or fate of the *Bacillus* component in field activity has been sadly neglected and requires further thought and effort. Also, fully developed international and national programs, integrating examination of new microbial agents in a logical series of stages, needs further development. In spite of the energy shortage we still need to protect man and his environment.

It is no longer a question of whether there will be bacterial field candidates, or even whether we will be ready for them as they appear in the next year(s). If Goldberg's estimate of 56.7 g/acre for SSII-1 proves to be correct we have a bacterial agent that is competitive with chemicals now.

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DISCUSSION

C. M. IGNOFFO: We are dealing with the aquatic environment, and I think it will become evident quite soon that the advancements in this area are not as great as those that have occurred in the terrestrial environment. People have been working with the terrestrial environment in terms of biological control agents, more specifically pathogens, for the last 25 years. So they have had an opportunity to build up a significant amount of baseline data.

S. R. DUTKY: One question, in regard to the numbers. It sounds simple to say you need 10^3-10^2 to 10^6-10^7 per milliliter, but there are a lot of milliliters in an acre-foot of water. So the statement that the organism does not develop in the host and kills it by magic otherwise is no advantage to the inoculation as compared with *B. popilliae* in a closed environment. Here you're using a very small dosage, relatively speaking, at intervals of high concentration, and thereby can use it at a level that will kill 50% of the contact insects in a month. Then you realign the build-up so that 10 years later you go back to the treated areas and find that the population of remaining spores in the soil is sufficient to give 10% immediate infection. The whole thing builds back up again quickly. Fields of that type have been under test since 1938, and they've been tested considerably since then.

JOHN BRIGGS: It's appropriate as we look at the theme of this meeting to appreciate that we have two individuals who have devoted an enormous amount of time in matters of this type. One is Dr. A. A. Arata, who is representing the World Health Organization and is an observer here, and the other is one of his predecessors, Dr. Marshall Laird, now at Memorial University of Newfoundland, who will appear on the program later this morning. Both men contribute significantly in bringing to bear the efforts now identified as consortia in the vector management business and in the matter of impact of biological and chemical agents on aquatic environments. The consortia are made up of national public institutions, universities, federal granting agencies, international public agencies, like FAO and WHO, and commercial groups throughout the world, frequently identified as the fermentation industry or industrial groups interested in propagation and formulation of biological agents. The consortia are evident, and I think Dr. Singer's remarks were pertinent in calling our attention to the activities of these consortia that we already see in work with *B. sphaericus*. We will hear from Dr. Laird later concerning some industrial groups and their activities with international agencies in Upper Volta, Africa, with respect to work on black

flies, a fine example of cooperative efforts, unprecedented in terms of public health.

Dr. Singer commented briefly about the review system for biological agents. This is analogous to the review system for chemical agents that has been observed for a number of years by WHO. I trust that Dr. Arata may have an opportunity to enlarge upon this subject later since it is quite appropriate to the subject of this entire conference, not only on the biological agents but on the chemical ones as well. In brief, we are dealing with a 5-step review system that begins with laboratory review, some presumptive safety examinations of biological agents, i.e., *B. sphaericus*, *Lagenidium* sp., *Coelomomyces*, and *Metarrhizium*.

In conclusion, Dr. Singer, there was one point mentioned which is quite pertinent to the whole review system. How does one positively identify an agent and distinguish it from other agents? A case in point is the reported pathogenicity of *B. sphaericus* to humans. When we attempt to identify or characterize something like *B. sphaericus*, we have already heard this morning that there are some "strains" that exceed by 10,000 X the activities of other "strains." It may be beyond the capacity of some groups to differentiate this strain difference by appropriate bioassays. Thus, we look at the conventional chemical, biochemical characteristics of microorganisms and morphological characteristics to identify species.

SINGER: I'd like to answer three things that were brought up. One, in terms of safety, two, in terms of how you measure activity, and three, the specific article by Farrar. These groups of organisms, morphological group 3 and morphological group 2, are very poorly understood and characterized. Now, what this all boils down to is, What is a species in procaryots (bacteria and viruses) compared with plants, animals, fungi, etc., which are, generally speaking, eucaryots? The concept of species in procaryots is poor. In the recent Bergey's Manual identification is to species, genus, family, period. What most microbiologists, or bacteriologists, consider as a species may be a closely related group of strains. This is a spectrum of organisms with similar or common characteristics. For example, in first dealing with *B. sphaericus*, we went to the American type culture collection and obtained *B. sphaericus*, *B. sphaericus* var. *fusiformis*, which is still a *B. sphaericus* (and it is *B. sphaericus* var. *fusiformis* that happens to hit the mosquitoes). The point is that we don't know too much about differences within this one species. This is not a question of species, for there are strains that hit the mosquito and strains that won't. Somebody might say, "It has a round terminal spore. It's *B. sphaericus*." Now, this fellow in Farrar's paper, who has everything in the world wrong with him, is said to be infected with *B. sphaericus*. This has not been proven, and I question whether there had been an adequate amount of work done.

The second thing is, how do you measure? In working with a non-obligate parasite, it's easier to set up an in vitro test, in terms of a huge number of insects per point or a screening type test like we do. It's easy to measure in terms of numbers per milliliter. When you go

into the field, with a product you want to use, it's measured in terms of amount of powder (or whatever the preparation is) per acre. *B. sphaericus* is only a year and a half old, thus, we have to have a model with which to measure. We've just chosen the convenience of organisms per milliliter--to give it a reference, to measure or compare *B. thuringiensis* (which we know a lot about) and *B. sphaericus*. Again, in terms of safety, my clinical friends tell me (in terms of this point) that bacteria as innocuous as *B. subtilis* can give galloping infections in many hospitals. Does this mean that *B. subtilis* is dangerous? It depends upon the strain we're working with.

IGNOFFO: I think the point is valid. There are many reports that get into the literature and become expanded beyond their original worth. This may inhibit development of a lot of these microbiological agents.

MARSHALL LAIRD: Just a question on the matter of safety testing. The honeybee and silk worm are well entrenched insects which one must look at. Bear in mind the kind of target areas that WHO, for example, might wish to test a *Bacillus thuringiensis* strain upon. In Southeast Asia, not infrequently the larval habitat has *Eichhornia crassipes*, or water hyacinth. The water hyacinth, in turn, is a harbor for mansonia mosquitoes that are filariasis vectors. A certain amount of work is in progress on the feasibility of using biological approaches in the integrated control of water hyacinths. One of the leading candidates is a lepidopteran and *B. thuringiensis* is well known to hit a very wide range of lepidoptera. I wonder whether that particular semi-aquatic moth might be regarded as a worthwhile candidate for safety testing in evaluating *B. thuringiensis* strains.

SINGER: If you recall, we tested *B. sphaericus* against the Indian meal moth, which is a lepidopteran (and one we could rear). It lacked activity, even against beetles. It's very obvious that you have to test against various kinds of non-target hosts, which hasn't been done, except in these few cases. It depends upon where in the world or in this country you want to go, which determines the non-target material that is needed to test against. For tests in these rice paddies, you will have to do this. But this is part of the considerations WHO is making in their scheme for testing biological agents.

B. W. DAVIDSON: I'd like to speak to Dr. Dutky's remarks on amounts necessary for field testing. Our preliminary work has shown that mosquito larvae are incredibly efficient at removing bacteria from the water. We found that very brief contacts, i.e., five minutes, with insecticidal *B. sphaericus* was sufficient for them to pick up enough to kill in LD_{98} amounts. Hopefully, in the field situation this would allow the insecticide to drift past them, and they might possibly pick up enough. It depends on how actively the larvae are feeding and if they are filter feeding with the rapidity and efficiency with which *Culex quinquefasciatus* appears to do in the laboratory. The second thing is

the question on the honeybees. It really depends on what part of the world you're working in. In Arizona, for example, almost all open water is honeybee water. They visit every open area where they can possibly find water. Honeybees in Arizona are extremely important because of the citrus industry. Thus, any time you treat water in an area of the world where water is in short supply, you're going to have honeybees picking up the insecticide.

A. A. ARATA: For quite a number of years now, we've had the very intensive program in screening of chemical insecticides. As John Briggs mentioned, this goes through several different stages, with the whole series collaborating with laboratories around the world and a number of WHO field research units who conduct actual field trials of the insecticides. When I left Geneva several weeks ago, we had passed the 2000 mark in number of particular compounds that were examined in this period of time. Of these, the vast majority were rejected in very early stages. A much smaller number have gotten into field operations. When I took over the responsibilities for biological control activities in WHO, it became apparent that we lacked any sort of coordination of this type in the biological control field, not only for a particular class of agents or a particular class of vector species, but rather across the board. Therefore, I've proposed an analogous scheme in five stages--it's only tentative, I've discussed it with various people. From our standpoint we see that this is a reasonable approach where, in the first stage, we are concerned with identification and characterization of the organism, its efficacy, and some indication as to its production in reasonable quantities. The second stage, of course, would be mammalian infectivity and, following some satisfactory results at this stage, we would like to review the status of the organism. Two WHO research units would probably receive the materials, the one in Kaduna, Nigeria, and the other in Jakarta, Indonesia. Here we have people concerned with biological control. In addition, we have many entomologists and others working on the whole population dynamics of the target species. So we know what's in the area, we have very good backup for the entomological assessment. Following satisfactory results on very small field trials, rice paddy plots or other situations as the environment demands, we would then consider going back into the laboratory, and concerning ourselves with the non-target species in depth. As has been brought out, a satisfactory field test in Indonesia or one in Africa would present a completely different spectrum of non-target organisms. Thus, we'd like to find out what the efficacy in the field is against the target before we concern ourselves with this huge spectrum that we might have to deal with. Otherwise, we've got the whole aquatic biota of the world with which to be concerned.

Following a limited field trial, should this prove satisfactory, and then testing against non-target organisms with perhaps even more detailed testing for mammalian and specifically human safety, then we can consider using the auspices of our WHO field units, and through them conduct larger-scale testing. This is proposed in the particular situation for *Aedes*, Culicine *Anopheles* and, hopefully, in the future,

with black flies and even triatoma bugs. Here we are examining the possibility of using insect parasites.

GEORGE ALLEN: We should take into consideration that we have two pests of the aquatic environment. One developing rapidly from the international standpoint in the last few years is aquatic weeds. In a recent meeting in India, UNESCO sponsored a program establishing the most important aquatic weeds in the world. As Marshall Laird pointed out, water hyacinth, *Eichhornia crassipes*, is foremost. One of the problems we are working on in Florida is this tremendous problem of aquatic weeds, on which we are spending about $5.5 million/yr. We are looking for many areas of biological control from two standpoints: the submerged weeds of what we call hydrilla, water milfoil, areas like this, then we're talking about the floating, or the type *Pistia* or water lettuce and water hyacinth. One of the major areas that we're putting a lot of money into is importation and/or utilization of natural enemies, primarily insects. Dr. Laird mentioned one lepidoptera against water hyacinth; we're looking at six, or potentially six, insects from Argentina on water hyacinth alone. One of the biggest problems that we've been faced with, from a pathological standpoint, is that they have disease problems of their own. One of the most promising insects we have is a lepidopteran on a water hyacinth. But there's no way that Fred Bennett, in Trinidad, can rear this insect for biological study because of a microsporidan parasite. We have found a lepidopteran that attacks water hyacinth in Florida. India is interested in bringing it into their country, but one of our biggest problems is getting the insect free of disease. We've got to be careful with any type of organism we put into a new environment.

RETO ENGLER: It has been brought out today that identification is quite important for safety considerations. I would like to add two things about the identification, namely, that since we are working with toxins, it would be important to find out what these toxins are, and their chemical nature. For instance, with *Bacillus thuringiensis* two toxins, the endotoxin and the exotoxin, have been described and could be used for pest control. Since one is proteinaceous and another is a nucleic acid analog, we have to approach their safety considerations quite differently.

Furthermore, if we are working with bacteria, I believe it would also be important to describe its antibiotic spectrum. Thus, we will have a means of interrupting any infection that should occur.

IGNOFFO: Thank you. We should be careful of another pitfall, and that is spending so much time trying to identify a compound. If that were a restriction prior to use, it might take as long as 25 years to find out what that protein complex is. In the meantime we've neglected a thorough developmental phase which could result in a replacement for a more toxic material.

USE OF VIRUSES FOR CONTROL OF AQUATIC INSECT PESTS

Darrell W. Anthony*

ABSTRACT

Virus diseases have been observed in many invertebrate species in aquatic and semi-aquatic habitats. These include *Baculoviruses* of the nuclear polyhedrosis (NPV) type, cytoplasmic polyhedrosis viruses (CPVs), entomopoxviruses, *Iridoviruses*, and other small nonoccluded icosohedral viruses. This report reviews the literature on laboratory and field studies concerning better known virus diseases of aquatic insects with special emphasis given to viruses pathogenic to mosquitoes. Infectivity, host range, specificity, and cross transmissibility are discussed as important factors in consideration of an entomopathogen for potential field use.

There are still insufficient research data on any of the viruses of mosquitoes, or other aquatic insects, to make definitive judgments regarding their use as biocontrol agents. A nuclear polyhedrosis virus affecting *Aedes sollicitans* shows promise for further research. Epizootics of this virus have been observed in natural mosquito breeding areas, and laboratory studies indicate that relatively high infection rates can be achieved. There are no commercially available preparations containing mosquito viruses, or other invertebrate viruses for control of aquatic insect pests. Furthermore, it is doubtful that such preparations can be developed within the next 5 years.

INTRODUCTION

The usefulness of virus diseases as an alternate method for controlling certain insect pests of crops and forests has been well known for many years. However, their effectiveness for management of aquatic insect pests has not been shown, thus insects such as mosquitoes and blackflies have been controlled primarily by extensive use of organic insecticides. Development of insecticide resistance in some insects and danger of environmental pollution by persistent insecticides has resulted in an intensive search for nonchemical methods of controlling these pests. There are a large number of viruses, protozoa, fungi, bacteria, and nematodes which may be useful as biological control agents in management of mosquito populations (9). The present paper deals generally with virus diseases known to occur naturally in aquatic invertebrates, with special emphasis to virus diseases of mosquitoes, and the

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factors that may affect their possible use as biocontrol agents.

Intensive study of insect viruses is a relatively young endeavor. The earliest reports referring to virus diseases in aquatic insects were from mosquitoes (14, 15, 18), however, the viral nature of these apparent infections was not confirmed. The first virus from an aquatic insect was described by Xeros (52) when he reported an *Iridovirus*[1] (Tipula iridescent virus [TIV]) from larvae of the cranefly, *Tipula palidosa*. Subsequently, a transmissible agent, believed to be a polyhedrosis virus, was reported from the mosquito, *Culex tarsalis* (33). Since that time, many virus diseases, including occluded and nonoccluded types, have been found in a wide variety of aquatic invertebrates. The types of virus diseases in aquatic invertebrates and some of the host groups from which these were recorded are noted below.

TABLE 1. Virus Diseases of Aquatic Invertebrate Organisms

Hosts	*Iridovirus*	*Baculovirus* (NPV types)	Near *Reovirus* (CVP types)	Entomopoxvirus	Other viruses[a]
Insects					
Coleoptera		+			
Diptera					
Ceratopogonidae	+				
Chaoboridae	+				
Chironomidae	+		+	+	+
Culicidae	+	+	+		+
Simulidae	+				
Tipulidae	+				
Trichoptera					
Limnephilidae		+			
Crustacea					
Malacostraca	+	+			

[a] Includes small nonoccluded icosohedral viruses, densonucleosis virus, and the tetragonal virus.

[1] Virus classification and nomenclature used herein is that given by: Wildy, P. 1971. Classification and Nomenclature of Viruses. Monographs in Virology No. 5. 81 p. S. Karger, Basel, Munchen, Paris, London, New York, Sidney.

In addition to the viruses in this tabulation, we have seen in our studies viral infections in Ephemeridae, Odonata, and in several additional groups of Crustacea. It is believed that further investigation will show that viruses are, indeed, quite common in many species of aquatic invertebrates.

The virus diseases of aquatic insects can be placed in two general groups, occluded viruses and nonoccluded viruses. The occluded viruses, as indicated by the name, have their virions formed within proteinaceous crystals, commonly referred to as inclusion bodies or polyhedra. These crystals range in size from 0.5 to several micrometers and can be readily recognized by phase microscopy or by conventional light microscopy from hematoxylin-stained preparations. Three types of occluded viruses are known from aquatic insects: *Baculoviruses* (nuclear polyhedrosis viruses or NPVs), cytoplasmic polyhedrosis viruses of CPVs (possibly allied to *Reoviruses*) and entomopoxviruses. Only the NPVs and CPVs occur with regularity in mosquitoes; however, an entomopoxvirus of mosquitoes is reportedly being studied in France (5).

The nonoccluded viruses include a large number of virus diseases in which the virions are not enclosed within proteinaceous crystals. Of those occurring in aquatic insects, the *Iridoviruses* are the best known. These viruses develop within the cytoplasm of infected cells and form paracrystalline arrays which may, by Bragg reflection, impact a distinctive iridescence when viewed by reflected light (35). The iridescent viruses (IVs) are the largest symmetrical viruses known, ranging from 125 to more than 200 nm in diameter. All that have been studied biochemically have been shown to be DNA viruses. The morphology, DNA content and the site of replication of iridescent viruses are similar in many aspects to other nonoccluded viruses of fungi, reptiles, amphibians, fish, and mammals. Therefore, Stoltz (41) has suggested that they all be referred to as icosohedral cytoplasmic deoxyriboviruses (ICDVs).

IRIDESCENT VIRUSES (GENUS *Iridovirus*)

Mosquito iridescent viruses (MIV) were first reported in larvae of *Aedes taeniorhynchus* from Florida (13) and from larvae of *A. annulipes* and *A. cantans* from Czechoslovakia (48). MIVs have now been reported from 13 species of mosquitoes as follows:

Aedes annulipes	Czechoslovakia (48)
A. cantans	Czechoslovakia (48)
A. cantans	Great Britain (46)
A. detritus	Tunisia (47)
A. detritus	France (30)
A. dorsalis	USA (Nevada) (7)
A. fulvus pallens	USA (Louisiana) (7)
A. sticticus	USA (Louisiana) (9)
A. stimulans	USA (Connecticut) (1)
A. taeniorhynchus	USA (Florida) (13)
A. vexans	USA (Louisiana) (7)

Psorophora ferox	USA (Louisiana) (7)
P. confinnis	USA (Louisiana) (5)
P. horrida	USA (Louisiana) (9)
P. varipes	USA (Louisiana) (9)

An MIV from *A. taeniorhynchus* was reported which produced a blue iridescence in patently infected specimens (51). This was designated as TMIV (T for turquoise) and the original isolate as RMIV (R for regular) (39). The two isolates were compared by electron microscopy and it was found that the TMIV was a distinctly smaller virus, averaging 160-175 nm compared to 185-195 nm for RMIV (2).

Observations on field infections indicate that the incidence of MIV in larval populations is very low, rarely exceeding one percent (1, 7). Also, laboratory studies have shown that only a relatively small percent of larvae exposed to MIV suspensions actually develop patent infections. Infection rates in larvae were demonstrated as high as 33% in some experiments, however, the average was only 4.4% (37). The average level of infection for 68 serial passages of RMIV in *A. taeniorhynchus* was 16%, although 60% infection was obtained in one test; for 30 passages of TMIV the average infection rate was 21% (51). Hembree (31), with the same mosquito species, obtained only slightly more than 10% infection in most of his tests. Transovarial transmission has been demonstrated by a number of workers (27, 37, 51). However, since all patently infected larvae usually die prior to pupation, only female larvae exposed to MIV during the late instars develop to the adult stage and transmit the virus to their progeny. Although the percentage of females that transmit the virus transovarially has been found to be 15% or less (27, 31), this mode of transmission does provide a mechanism for perpetuation of the virus in nature. Thus, a tentative cycle of natural transmission has been proposed (38). The latter workers state that transovarial transmission produces infected larvae which die in the fourth instar. These larvae provide a source of new infection when healthy larvae feed on the diseased cadavers prior to pupation. This in turn leads to the presence of infected adults which complete the cycle by depositing infected eggs.

Larvae from MIV-infected eggs of *A. taeniorhynchus* and *Psorophora ferox* stored for 26 weeks became patently infected (51). This time interval is believed sufficient for survival of the disease in nature during long droughts and periods when the eggs are in diapause (5).

It is of interest that the 13 species of mosquitoes that are hosts of MIV all belong to the floodwater genera *Aedes* and *Psorophora* which have diapausing eggs. MIV seldom if ever occurs in genera of mosquitoes which lack diapausing eggs (9). All attempts to transmit MIVs to non-floodwater mosquitoes have failed. For additional information on MIVs and other virus diseases of mosquitoes the reader is referred to the excellent review of the subject by Federici (22).

Since the first description of an iridescent virus from *T. palidosa* (Diptera: Tipulidae) (52), *Iridoviruses* now have been reported from several other aquatic insects. Larvae of *Culicoides arboricula* (Diptera:

Ceratopogonidae) in two tree holes were reported infected with an *Iridovirus* (9); 50% of the larvae in 17 collections made from one tree hole in about one year were infected. Another iridescent virus was described from larvae of *Corethrella brakeleyi* (Diptera: Chaoboridae) (6). The average infection level noted in field collections during that year was 36.1%, from a low of 0% in August to 70% in November. A virus disease was described in *Chironomus plumosus* which was obviously a member of the *Iridovirus* group but which did not produce a characteristic iridescence in the infected larvae (44). We have observed similar virus diseases in *Goeldichironomus holoprasinus* and in *Chironomus attenuatus* from material collected at Gainesville, Florida. The only record of an *Iridovirus* in black flies was reported in 1968 (49). While the disease produced a violet iridescence in *Simulium ornatum* (Diptera: Simuliidae), the author concluded that the disease in black flies is rare. In our studies at Gainesville we have observed iridescent types of viruses in several groups of Crustacea including daphnids and amphipods. However, we have had no opportunity to study these diseases.

Other nonoccluded viruses occurring in aquatic insects include small icosohedral viruses, 50-70 nm in diameter, that have been observed in Chironomids. One of these viruses has been reported in *G. holoprasinus* (20). The nerve, muscle, fat body, and gut were infected.

A densonucleosis virus has been described from a laboratory colony of *Aedes aegypti* (36). Neither this or other similar viruses have been reported in naturally occurring mosquito populations.

The virus disease first observed in larvae of *Culex tarsalis* was thought to be a "possible polyhedrosis virus" because of the tetragonal-shaped crystals observed in infected cells (33, 34). Ultrastructure studies at our laboratory and more recent studies (43) indicate that the tetragonal crystals observed by light microscopy are not proteinaceous bodies containing virions as is the case with NPVs or CPVs. Instead, the electron micrographs show crystals composed of very small particles in a rectilinear array, which appear to develop in the nucleus of imaginal disc cells and epidermal cells and then invade their cytoplasm. Unequivocal proof of the viral nature of the particle is lacking (5). However, the disease is transmissible, and similar types of inclusions are reported in larvae of *Aedes sierrensis* and *Anopheles freeborni* (33). This disease was also reported in larvae of *Culex salinarius* from Louisiana, and in larvae of *Anopheles crucians* from the same area (8).

NUCLEAR POLYHEDROSIS VIRUSES (GENUS *Baculovirus*)

The virions of the nuclear polyhedrosis viruses (NPVs) are rod shaped, 200-400 nm in length by 68-80 nm in diameter, and are occluded in proteinaceous inclusion bodies of varying sizes and shapes. All NPVs that have been studied from the biochemical and biophysical standpoint have been shown to be DNA viruses. In mosquitoes, infections are confined to the epithelial cells of the midgut and gastric caecae. The disease can usually be recognized by examination with a dissecting

microscope. When infected larvae are examined against a black background, greatly hypertrophied, white nuclei can be observed through the cuticle of the living larva. As the infection progresses, the entire midgut and gastric caecae may become white and hypertrophied.

Since the first confirmation of an NPV infection in mosquitoes which was found in *Aedes sollicitans* in Louisiana (11), NPV infections have been found in field-collected larvae of several additional species.

Aedes taeniorhynchus	Louisiana (5)
A. triseriatus	Louisiana (5)
Anopheles crucians	Louisiana (12)
Culex pipiens quinquefasciatus	Louisiana (5)
C. salinarius	Louisiana (12)
Psorophora confinnis	Louisiana (12)
P. ferox	Louisiana (5)
Uranotaenia sapphirina	Florida (Hazard, personal communication)
Wyeomyia smithii	Massachusetts (28)

In most instances, the nature of these NPV infections has been confirmed by electron microscopy.

NPV from *A. sollicitans* could reportedly be transmitted perorally, and the disease was shown to be highly lethal (11). Laboratory transmission tests yielded infections in only about 5% of the exposed larvae. A naturally occurring viral epizootic in larvae of *A. sollicitans* was recorded in southwestern Louisiana (12); 70% of the population was infected. This epizootic involved a CPV as well as an NPV virus. Additional epizootics approaching 70% infection levels were reported (8). Laboratory studies showed that the NPV from *A. sollicitans* was transmissible to *A. triseriatus* and studies on the pathology of this virus in *A. triseriatus* have been reported (23, 24). An NPV infection larvae of the pitcher plant mosquito, *Wyeomyia smithii*, has recently been reported (28). This virus is similar in size and appearance to other *Baculoviruses* of mosquitoes; however, the formation of polymorphic inclusion bodies appears to be a unique feature.

An NPV from a caddisfly, *Neophylax* sp., was described in which the only external sign of disease was the transparent appearance of the infected larva due to the atrophied condition of the fat body (29). Healthy larvae had a well-developed fat body and were milky-white in color. Light and electron microscopy showed the midgut to be the site of infection, and the authors note that the virions were similar to other NPVs.

CYTOPLASMIC POLYHEDROSIS VIRUSES (POSSIBLY ALLIED TO *Reoviruses*)

Diseases of mosquitoes caused by cytoplasmic polyhedrosis viruses or CPVs have been recorded in at least 19 species within 9 genera (22). In the diseases that have been studied, these viruses also appear to attack only the midgut and caecae of mosquitoes.

The CPVs are occluded viruses that are icosohedral in shape, usually ranging from 50 to 70 nm in diameter. The inclusion bodies may be small and cuboidal in shape (3, 4, 16), or of varying size and irregularly-shaped (11, 12, 25). The CPVs of mosquitoes are believed to be double-stranded RNA as is the case with CPVs in other insects.

The CPV virus in *Aedes sollicitans* can be recognized by the yellowish-white appearance of the posterior half of the affected midgut and gastric caecae (10). These workers further indicated that although the gut cells become so pendulous that they burst under the slightest pressure, heavily infected larvae usually were able to pupate and emerge as apparently healthy adult mosquitoes. Transovarial transmission of this virus was noted in *A. sollicitans* (12). CPV in adult *A. taeniorhynchus* which survived patent infections as larvae was also observed (25).

Observations have not shown a high incidence of CPV infections in the field; however, most mosquito species are hosts of a CPV (5), and the infections usually are not lethal to infected larvae. An exception to the observed low incidence of natural CPV infections has been reported (12). This study showed a high incidence of CPV associated with a NPV infection in *A. sollicitans* in southwestern Louisiana. It was postulated that the CPV may have been a predisposing factor for the extremely high incidence and lethality of the NPV infection.

Other aquatic insects known to be hosts of CPVs include several species of Chironomidae. A CPV was described from small numbers of third instar larvae of *Chironomus plumosus* during laboratory colonization of this species (43). Two distinctly different CPVs have been described from *Goeldichironomus holoprasinus* (23) and from a *Chironomus* sp. collected from greenhouse basins at the USDA Insect Attractant, Behavior and Basic Biology Research Laboratory at Gainesville, Florida. Recently another CPV, different from those described earlier, has been seen in a *Chironomus* sp. In all of these infections, the midguts of the larvae appeared to be the primary area of infection. In an "added note of proof," the disease was reported found in a field-collected fourth instar larva from Lake Winnebago, Wisconsin (42). These workers also reported the existence of another CPV which was restricted to the fat body.

Patently infected larvae of *G. holoprasinus* with a CPV collected from greenhouse basins usually died within 24 to 48 hr after collection (23). Laboratory studies showed that the virus could be transmitted perorally, and infection rates as high as 40% were achieved.

ENTOMOPOXVIRUSES

Entomopoxviruses have been recorded from several species of Lepidoptera and Coleoptera and from a single orthopteran (45). However, the only aquatic insects from which these viruses have been reported are chironomids. The following species are known to be hosts:

Camptochironomus (chironomus) tentans	Czechoslovakia (50)
Chironomus luridus	Germany (26)
C. luridus	Germany (32)
C. attenuatus	USA (Texas) (45)
C. plumosus	(45)
Goeldichironomus holoprasinus	Florida (22)
Procladius sp.	Florida (Anthony, unpublished)

The disease was studied in *C. luridus* and the fat body was reported to be the primary site of infection (32). The disease was described in *C. attenuatus* as a hemocytic poxvirus (45). At Gainesville, we have examined many infected specimens of *G. holoprasinus*, and it is also our opinion that the hemocytes are the probable site of infection in this species. The virus is highly lethal to *G. holoprasinus*. The disease becomes apparent in late instar larvae and all patently infected specimens die before pupation. The virus has been transmitted back to *G. holoprasinus* perorally in the laboratory, but in our tests, infectivity level was low and quite variable.

HOST SPECIFICITY AND CROSS TRANSMISSION STUDIES

IRIDESCENT VIRUSES

MIV has never been found in natural populations of *A. sollicitans*, although larvae of this species are frequently found breeding with patently infected larvae of *A. taeniorhynchus*. However, in the laboratory, RMIV has been transmitted from *A. taeniorhynchus* to larvae of *A. sollicitans* and *A. vexans* (51). All rates of transmission were very low (less than 1%) and much lower when the MIV was transmitted back to its normal host. These workers also transmitted the MIV from *P. ferox* to *A. taeniorhynchus* and *A. vexans* and, as before, transmission rates were very low. Additional cross transmissions include:

MIV from *A. taeniorhynchus* to *A. nigromaculus*
MIV from *P. ferox* to *P. varipes*
MIV from *P. ferox* to *P. horrida*
MIV from *P. horrida* to *P. confinnis*
MIV from *P. confinnis* to *P. horrida*

All rates of transmission were low (Chapman, personal communication), and all attempts to transmit MIVs to non-floodwater mosquito species have proven futile.

Apparently there have been few attempts to transmit the *Iridoviruses* from mosquitoes to other hosts. Attempts at the Gainesville laboratory to transmit RMIV to *Heliothis zea*, *Trichoplusia ni*, and *Galleria mellonella* by feeding heavy virus suspensions were unsuccessful. Intrahemocelic inoculation of purified virus into *G. mellonella* resulted in death to all larvae within 72 hr after injection. Electron microscope studies of the specimens failed to show evidence of virus replication.

The successful transmission of the *Chilo* iridescent virus (originally from the lepidopteran, *Chilo supressalis* but maintained in *G. mellonella*) to 13 species of mosquitoes has been reported (25). Even though transmission levels were generally low, it is highly significant that species of *Anopheles*, *Culex* and *Culiseta* (which were refractive to infection by all MIVs) became infected with this virus.

An iridescent virus from the grub, *Sericesthis pruinosa* (SIV) was transmitted to *Aedes aegypti* (17). Two larvae developed a blue iridescence two weeks after exposing early instar larvae to media to which SIV had been added. It was concluded that the larvae were infected with SIV.

Tipula iridescent virus (TIV) apparently has a wide host range (40). This virus has been experimentally transmitted (mostly by the inoculation of virus material) to 12 species of Lepidoptera, 3 of Coleoptera, and 7 species of Diptera. The susceptible Diptera include: 4 species of *Tipula*, *Bibio marci*, *Calliphora vomitora* and a *Mycetophila* sp. No attempts to transmit this virus to mosquitoes are noted.

The iridescent virus from the chaoborid *Corethrella brakeleyi* is cross-transmissible to *C. appendiculata*; however, early instar mosquito larvae of *A. sollicitans*, *A. taeniorhynchus*, and *P. ferox* were not susceptible to this virus (6).

In tests with the iridescent virus from the chironomid, *G. holoprasinus*, Hazard (personal communication) found that the virus was transmissible back to larvae of *G. holoprasinus* but not to *Chironomus attenuatus*, or to the mosquitoes, *A. aegypti* and *Anopheles quadrimaculatus*.

Transmission studies with the tetragonal virus isolated from *C. salinarius* were reported (10). It was found that this virus was transmissible to larvae of *C. tarsalis*, but transmission trials with *C. pipiens quinquefasciatus*, *C. peccator*, *Culiseta inornata*, and *A. taeniorhynchus* were negative.

NPVs

Because of the limited amount of virus material available from the isolated collections of infected larvae, only a few transmission studies have been conducted with the NPVs from mosquitoes. The NPV from *A. sollicitans* has been transmitted to larvae of *A. triseriatus*, *A. aegypti*, *A. nigromaculus*, *A. tormentor*, *P. ferox*, and *P. varipes* (5). Species of *Culex* and *Anopheles* were not susceptible to this virus. Hazard (personal communication) was unable to transmit the NPV from *Uranotaenia*

sappharina to either *A. aegypti* or *Anopheles quadrimaculatus*. There have been no tests with mosquito NPVs and nontarget organisms.

CPVs

Although CPVs have been recorded from at least 19 species of mosquitoes, there is a great lack of knowledge in regard to their infectivity to other species of mosquitoes. Nothing is known regarding their ability to infect nontarget organisms. The CPV from larvae of *C. salinarius* was transmitted to larvae of *C. territans* and *Culiseta inornata* but not to *A. sollicitans* (5). A CPV from the larvae of *A. sollicitans* was transmitted to *A. taeniorhynchus* and *P. ferox*, but this virus failed to infect larvae of *C. salinarius* (12). This may indicate that the two viruses are distinct. Hazard (personal communication) found a dual infection of NPV and CPV in *U. sappharina* and successfully transmitted the CPV to *A. aegypti* but not to *Anopheles quadrimaculatus*. As noted earlier, the NPV did not infect either of the test species.

Infection trials with CPVs from chironomids have shown that the CPV from *G. holoprasinus* was not infective to larvae of *C. attenuatus*; similarly, the CPV from *C. attenuatus* was not infective to larvae of *G. holoprasinus* (Hazard, personal communication).

ENTOMOPOXVIRUSES

During 1972 and early 1973, we observed a mixed population of *C. attenuatus* and *G. holoprasinus* breeding in an aeration pond at the University of Florida Sewage Treatment Plant at Gainesville, Florida. *C. attenuatus* was by far the predominant species in this population, but the entomopoxvirus was found only in *G. holoprasinus*. Infectious levels in *G. holoprasinus* were usually quite low (1% or less), but the virus was found in nearly every collection made over a period of several months. The virus from *G. holoprasinus* was very similar in size, morphology, and tissue specificity (20) when compared with the virus reported by Stoltz and Summers (45) in *C. attenuatus*. However, it may be a different virus as attempts to transmit it to *C. attenuatus* in the laboratory have never been successful. We also have recorded an entomopoxvirus from the larvae of a *Procladius* sp. collected from the Waccassassa River, about 30 miles west of Gainesville. From the ultrastructural standpoint, this virus appears to be identical to that from *G. holoprasinus*.

FIELD RELATED STUDIES WITH MOSQUITO IRIDESCENT VIRUS

Laboratory experiments (37) showed that cadavers of *A. taeniorhynchus* patently infected with MIV were a good source of infection to susceptible larvae. Field related studies (38) were then conducted to discover how the virus was acquired and perpetuated in nature. These workers conducted several types of experiments to (1) demonstrate infection rates in natural habitats after addition of infected larvae or cadavers,

(2) study the infectivity of the virus after storage in artesian water and 10% sea water, and (3) determine the persistence of the virus on sod after varying periods of time. The field studies were conducted by placing the experimental larvae (infected and noninfected) in large galvanized cylinders sunk into the side of swales. In some tests, the effects of the virus were evaluated against both first and second generation mosquito larvae. The first generation larvae were those that developed patent infections after exposure to the virus. Second generation larvae were the progeny of individuals exposed to the virus, but did not become patently infected.

Tests showed that live patently infected larvae (which would die within 24 hr) or macerated larvae provided a source of infection in the field. Infection levels ranged from a low of 1.4%, using an inoculum of 5 live infected larvae, to a high of 9.9%, using 100 macerated infected larvae. Comparisons were made of the infectivity of virus suspensions stored in artesian water and 10% sea water to first and second generation *A. taeniorhynchus*. In all cases infectivity rates in the first generation decreased with the storage period of the virus. This was especially obvious with virus stored in artesian water, where infectivity rates fell from 15.6% to 2.5% in the first 2 days. The virus remained active longer in sea water, producing infection after 20 days of storage, whereas there was no infection in artesian water after 10 days. In the second generation, infection rates were generally lower than in the first, and again, the loss of infectivity occurred sooner after artesian water storage.

Studies on the persistence of the virus were conducted with small pieces of sod which had been sprinkled with a suspension of 5 macerated infected larval cadavers in artesian water. When the pieces of sod were flooded and susceptible larvae added, all infectivity (to both first and second generation mosquitoes) was lost after 2 days of storage. It was indicated that the chance of larvae becoming infected in nature by virus liberated from the soil on flooding was very small. Under natural conditions, the virus liberated into floodwater probably would become uninfective before the breeding area became dry. The likelihood of its surviving even a few days between successive flooding seems remote.

Linley and Nielson (38) concluded that in nature the greatest number of individuals become infected through direct feeding of larvae on cadavers or fragments thereof. Larvae acquiring the virus during their early instars develop patent infections and die, thereby serving as a source of virus for older larvae which may acquire the disease in the late instars and transmit the virus to their progeny, thus perpetuating the cycle.

FIELD RELATED STUDIES WITH MOSQUITO POLYHEDROSIS VIRUSES

A viral epizootic involving both a nuclear polyhedrosis virus and a cytoplasmic polyhedrosis virus was studied in natural populations of *A. sollicitans* in southwestern Louisiana (12). The affected population was followed through three periods of flooding, and laboratory studies were conducted to aid in understanding of events that occurred in the field. Infective material also was introduced into an area having a very low infection rate to determine whether the rate of infection could be increased.

In the study it was noted that a series of overlapping broods of *A. sollicitans* may lead to a significant buildup of infective material within the habitat. A rise in the infection level from 8.6% to 70.8% was observed during a 10-day period when two successive broods of mosquitoes occurred. During the following 9 days there was no rain and the soil became parched and cracked because of the intense heat and sunlight. Rain brought forth another brood of mosquitoes, but at this time the infection level never exceeded 4%.

Another area was studied where these virus diseases occurred at frequencies of less than 0.1% of the population sampled. The authors selected a pond (10 x 20 ft x 8 in.) in this area and "seeded" it with 5 g of freeze-dried infected larvae collected at the site of the viral epizootic described earlier. After treatment, collections from this pond showed a significant increase in infection levels from less than 0.1% to 16.3%. The virus persisted in the area through at least two more broods at infection levels of about 5%, after which it dropped to its original incidence of less than 0.1%.

An evaluation of the separate roles of the two viruses was precluded by the presence of an unknown number of double infections. Laboratory tests showed that the CPV was not usually fatal; however, patent NPV infections were invariably fatal. It was suggested that the CPV may predispose the larvae to NPV infection and that the dual infection may bring about a synergistic effect. It was also pointed out that oil pollution at the site of the viral epizootic may have acted as a stressing factor.

DISCUSSION

It is apparent from this review that we know very little about the viruses that cause disease in aquatic insects or in other aquatic invertebrates. Occurrence and host records have been compiled, and some information has been accumulated on the pathology of a few of the viruses. In the case of the better known iridescent viruses, there is data on their biochemical and biophysical characterization. Although host range data are available for some viruses, they are not complete, and essentially nothing is known in regard to total ecology or the

possible effect of these viruses on nontarget organisms. The studies by Linley and Nielson (38), using sod that had been contaminated with MIV, indicated no infective residue after a one-day storage period. Similarly, the NPV/CPV epizootic in *A. sollicitans* diminished rapidly after the habitat became dry (12). Both studies suggest that the low incidence of virus infection seen in nature is maintained by a cycle of lateral (larva to larva) and transovarial transmission. Other than the tests and observations cited previously, there have been no experiments with viruses of aquatic insects to show their persistence in soil, runoff water, or in different types of water and habitats.

More basic research is needed before definitive conclusions can be made on the potential use of these viruses for control of aquatic insect pests. For instance, production of sufficient quantities of virus material for field experimentation, nontarget organism, and persistence studies has been a major problem. The science of insect virology is still very young, and although we have found a few viruses in aquatic insects, there probably are many more yet to be discovered. It seems possible, and quite probable, that a future undiscovered virus may have far greater potential for field use than any of those discussed in this report.

CONCLUSIONS

There is no commercially available preparation containing a mosquito virus, or any other invertebrate virus for use in control of aquatic insect pests, and it is extremely doubtful that any preparations can be developed within the next 5 years. Research has not yet progressed to the point where a particular virus can be selected as a promising candidate for further development. Of the virus diseases discussed here, the NPV from *A. sollicitans* appears to offer the greatest potential for future research. This virus (1) can be propagated in the laboratory with relatively high infection rates, (2) is lethal to all patently infected larvae, and (3) has been observed in epizootic proportions in field populations of mosquitoes. However, a concentrated effort of expanded research is needed before this virus can be developed for use as an effective agent for control of mosquitoes.

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DISCUSSION

T. B. CLARK: First of all, with respect to the tetragonal virus in mosquitoes, *Culex tarsalis* and *Culex salinarius*, I recently found a similar virus in *Culicoides*. This adds another family to your list of aquatic viruses, and the syndrome in *Culicoides* is almost identical to that in mosquitoes. I originally thought it was the same virus and tried unsuccessfully to transmit it to *Culex tarsalis*, which is a susceptible host to the tetragonal virus, and to *Culex quinquefasciatus* and *Aedes dorsalis*. I also inoculated a group of flies and larvae of the wax moth and navel orange worm without success.

The electron micrographs of that virus, however, indicate that the viral particle is just about twice the diameter of the one in mosquito. There is no doubt in my mind that the one in the mosquito is a virus of the same type. Secondly, I would like to mention the reference to a rectilinear array in the *Culex tarsalis* tetragonal virus crystal. I have some of Dr. Anthony's electron micrographs that show both hexagonal array and rectilinear array and both also appear in *Culicoides* virus.

Our original observations on the nuclear polyhedrosis of *A. sollicitans* were made in a Louisiana pasture situation in which the flooding was dependent on natural phenomena such as rains, winds, and high tides. Often, periods of several weeks would occur between rains, the habitats would get very dry, and the virus would apparently disappear. The pasture mosquito, *Aedes nigromaculus*, found to be susceptible to that virus, is found in the San Joaquin valley of California which is almost entirely cultivated by regulated irrigation. This mosquito is undoubtedly the most important pest species in California and it is also, unfortunately, the most resistant to insecticides. We conducted a simulated field study in 4 one-thousandth/acre ponds on the campus of Fresno State University during the last half of July and through early August. At that time of year, the normal daily maximum is about 101 F, and it tapers off, dropping to 99 F through the first part of August. We noted a decline in infection levels over three successive floodings of these small ponds, each one separated by three days. Mosquito abatement personnel wouldn't let us allow any mosquitoes to emerge so we would fill the ponds to about 4 inches of water, add about 2,000 newly hatched *Aedes nigromaculus* larvae, and then add some virus material. At that temperature in the San Joaquin valley we had pupae and emergence by 4 days, thus there was very little time for a virus to infect. On the fourth day we pumped out the water and screened it to recover any remaining larvae or pupae. We then crushed these, returned them to the pond and allowed the pond to dry for 3 days. We did this three times, of which the typical example is that on the last day, the fourth day of the first test, 42.2% of the larvae showed patent infection. In the next series of tests, the infection rate dropped 20%, that is, without adding any viral material. At the end of the second flooding, the infection rate was 22% and at the end of the third flooding it was 23%. The studies were terminated in late August, and the ponds not reflooded

until the following May. At this time we conducted the same tests, except that fresh viral material was not added. The ponds were filled with water and about 3,000 larvae were placed in each. No viral infections were detected in examination of most of those 3,000 larvae. It was concluded that survival of the virus over that length of time and in that situation was very poor.

We have also done some feeding experiments of virus-infected mosquito larvae to non-target species (none of this is in the literature); some of these non-target organisms, such as dytiscid and hydrophylid beetles, mature Odonata, and dragonfly and damsel fly larvae, were particularly good predators. We also tested *Gambusia*, our important mosquito control predator in California. To date, we have found no evidence of any infections or mortality resulting from these exposures. The *Gambusia* were tested in 10-gallon aquaria, about a dozen in each, and fed infected mosquito larvae over several months without evidence that disease could be transmitted in that way.

The real barrier to use of this virus in mosquitoes is the dosage required. The amount of virus required is really quite fantastic, at least with our present techniques of application. Quite possibly, this could be improved significantly using other application methods or protectants on the inclusion bodies. There is a fairly typical dose response of *Aedes nigromaculus* in terms of milligrams of polyhedral inclusion bodies per liter of water. Infection tests show that almost 10 mg of inclusion bodies per liter of water are needed to achieve close to 100% mortality. Let me break this down for you: About 2% of the dry weight of a patently infected or moribund fourth instar larva is composed of inclusion bodies. Thus, it takes 28 patently infected larvae or moribund fourth instar larvae to produce one milligram of virus. At this figure, taking 10 mg--of course, this is infecting the larvae as third instars and harvesting the virus from the fourth instars (if you expose larvae in earlier instars, you can get much better results)--at this dosage rate, according to my figures, it takes something like 12 pounds per acre to kill the larvae. That's not exactly practical.

This virus is one of the few organisms that I have worked with over a rather disappointing 13 years that has really done anything to mosquitoes in measurable quantities. Since the virus develops in the field, we have produced, in ponds, a 100% mortality within a 2-day period, so the virus is lethal, if you could get it there in the right quantities. Perhaps with protectants, or some way of extending the life of the viral particle and a better method of applying it so you don't have to worry about environmental saturation, it's quite possible that it could be useful under some circumstances.

JOHN PASCHKE: As far as our work with MIV is concerned, we're not overly enthusiastic about its use as a biological control agent, and I think that's been pointed out by Dr. Chapman a number of times. In connection with what Dr. Anthony said regarding the propagation and production, we have had success in getting infection in a number of tissue culture cell lines using both R and T type MIV. However, I'm not

encouraged about the production of the virus in the cells because it does not appear to be an economical means of propagation. We haven't really scaled this up so I don't have any concrete data--it does appear that the tissue culture, at this point anyway, would not produce enough virus to be practical. Furthermore, if we allow these cell lines to go for 2-1/2 days, the virus elutes from the cells and picks up a portion of the plasma membrane of the cells in which the virus was produced, and as nearly as we can tell, renders it uninfective to surrounding cells. Bioassays of the virus conducted on early instar mosquito larvae also appear to give negative results. I would assume that what we are looking at is a virion wrapped in plasma membrane from the host cell, the adjacent cells recognize that as host cell and viral infection doesn't take place. We stripped the outer coat from the virus and tried to inoculate virus cores into these cells. Apparently the chemical process of removing the outer coat eliminates the infectivity of the core, so that method doesn't look like a practical approach to infecting tissue culture cells.

Insect virology has been aimed mainly at biological control of pest species and I think many of us have felt that the insect viruses are rather unique in that they have this inclusion body that Dr. Ignoffo touched on. Incidentally, Dr. Ignoffo, you didn't talk about the nucleic acid being different. This uniqueness seems to be disappearing rapidly, as Dr. Couch has found a NPV in shrimp. Earlier we thought NPV's were quite unique, and that the inclusion body viruses in general were very unique. Obviously they're not as unique as we used to think they were. The icosohedral deoxyriboviruses (which include the iridescent viruses) have been shown to infect a number of animals, including vertebrates and plants. We also have representatives from the Picorna virus group, the Parvo virus group, and the pox viruses which have been mentioned; it seems these viruses are found in insects as well as in other animals and plants. This dictates that we have a very thorough biochemical/biophysical characterization, to substantiate species, or, if you prefer, to separate one virus from another. We need to carefully conduct quantified studies in cross-transmission to other insect species, but in this instance we are talking about the aquatic environment where there are many other species of animals which need to be carefully scrutinized. In some situations we may want to conduct comparative studies with other animal viruses.

C. M. IGNOFFO: One comment: I'm sure there is probably no living organism, if enough search is made, that would not harbor a viral entity. And as in many of the bacteria, the morphological distinguishing characteristics are not that distinct. Further clarification will probably have to be done on a much lower level. I'm sure you are aware of what's going on in an attempt to make the nomenclature and classification or taxonomy of viruses universal. There are many different types of viruses placed in the same groups--for instance, plant viruses, amphibian viruses, avian viruses, mammalian viruses, and insect viruses. These are done initially on the basis of morphological characteristics of the

virion and then further differentiation into its biochemistry.

H. C. CHAPMAN: Just about everything has been said on the aquatic viruses that we know. But I think we've got a long way to go, for we know very little. Hopefully, as Dr. Anthony has said, there are some better viruses than the NPV's that we found and worked with.

The virus in *A. sollicitans* seems to be about the best one we have and it's not too good. We have seen at least three or four epizootics in the field that have reached around 60-70%, which is pretty fair. Of course, in Louisiana that doesn't affect too many mosquitoes--the remaining 40% will kill you. In the last ten years we've learned a lot, but with the few people we have and the little money that we have working in this field, I agree that probably it'll be 5, 10, or 15 years before we can come up with a good candidate, not necessarily in a virus, but in biological control in general.

J. N. COUCH: I am mainly concerned, being a member of EPA, with aspects of possible effects on non-target species. However, as we've heard, very little is known about impact on non-target species. There are no publications available of reports of testing. I've searched the literature several times and spoken with many people in this area and very little has been published about testing these viral agents. I hope that data in people's files on tests that they've carried out on non-target species would be made available. These data, either positive or negative, will be of great value to people in EPA. Dr. Anthony's list in his first slide showed some of the viruses which have been found in aquatic organisms, particularly the insects. I would like to add some other published works that may be of interest to people here. In 1963 Dworky et al. published a paper in *Nature* describing a rod-shaped virus isolated from a microanalid. This was a very interesting relationship in that the microanalid, when cultured in an aqueous phase, produced tumor-like lesions. Virus-like, rod-shaped particles were isolated from these lesions only when the microanalid was cultured in an aqueous phase. These workers did not comment on the affinities of the virus except to say that in some respects it was similar to certain members of the Baculoviruses, ordinarily known only as nuclear polyhedrosis viruses. Also, in 1972, Viriton in France reported a nonoccluded but rod-shaped virus from the whirligig beetle. This virus had many characteristics of what we currently call Baculoviruses, but did not appear to produce mortality in infected specimens--at least none were observed. His conclusion was that perhaps this was an enzootic, or latent infection that rarely produced natural epizootics. Recently, at the Gulf Breeze laboratory, we have found an apparent NPV in pink shrimp, one of the animals we use in toxicity testing. Occluded and free virus occur in the hepatopancreatic cells of the shrimp, and it was found during the course of toxicity studies of chemicals using shrimp as a test animal. You will be able to visit my laboratory if you wish, and we have a display there of micrographs available to people who are interested in examining this in greater detail.

One future area that may pose some concern is the possibility of interactions between pollutant chemicals or chemicals in the environment and viruses. We have found in preliminary tests that when pink shrimp have been exposed to polychlorinated biphenyl Aroclor, apparently this chemical stress may elevate the prevalence of the virus in sample test populations. Rockendorfer and Denton (1973), in their work on invertebrate pathology, reported that they had achieved a nine-fold increase in virulence of a NPV by exposure of the virus to treatment with 3-methylcolanthrine, a carcinogenic drug. We have environmental factors here that have not been considered in great detail in the past but will be of concern in the future. Two other factors on which there are data published are the effects of temperature and salinity of the environment on NPV's.

IGNOFFO: I might direct one question to Dr. Couch. In this NPV found in pink shrimp, were you able to establish Koch's postulates, were you able to transmit and recover?

COUCH: We're working on this presently. The virus actually was only found about a year and a half ago. It has been consistently refound in wild shrimp as well as in those exposed to the chemicals.

R. B. JAQUES: I'd like to ask, Dr. Couch, do you know whether this virus is closely related to the insect virus? Do you know whether there is any relationship between this and the insect virus?

COUCH: I have no evidence that it's related to any of the known NPV's of insects. There appear to be some differences in polyhedral body substructure, etc., in this virus as compared with some of the insect viruses. However, I don't think this is too surprising when you look in a crustacean or some of the other arthropods (other than insects) to find many of these virus groups. As people become more interested, further developments are anticipated. Dr. Hazard and Dr. Anthony have indicated to me that they have found viruses of other types in crustacea, some fresh-water.

E. I. HAZARD: The viruses that we found in fresh-water crustaceans in Florida are of the icosohedral (ICDV) type, in daphnids and copepods; we've not seen anything at all similar to CPV's or NPV's.

D. W. ROBERTS: There are three types of rod-shaped viruses that have been reported. The Baculoviruses, the Pox viruses, and the Rhabdoviruses. The latter are rod-shaped in the general sense that they are bullet-shaped. There are many reports in virology and plant pathology of filamentous long rod-shaped particles. So the occurrence of a rod is not unqiue. It's been reported in the literature for a long time. I think initial reports were in plant virology.

A. M. HEIMPEL: I'd like to say that the occurrence of a DNA-bearing rod is unique, and it's unique in the viral kingdom. It may not be specific in lepidoptera to use that term "unique" in terms of differences between viruses, and the NPV's are unique within the virus group.

ROBERTS: The point I wanted to speak to is specificity. I've taken some flak in the years I have worked with the pox virus in insects because people find this a very loaded term. I would think that if you wanted to expose yourself to a virus, this group would probably be one of the safer ones, myxomatosis being an example of one of these viruses. At our institute in recent months, we have been frustrated by a pox virus we produce in large amounts. It comes from an insect, and has several characteristics in common with the vertebrate pox virus. It has the same enzyme complement, the general morphology, general morphogenesis, general size, all these parameters except host range, that seem to place it with the known pox viruses. We would like to do some basic biological comparisons for known characteristics of pox viruses. All pox viruses except one are known to reactivate any other pox viruses; that is, if you kill one with heat, and then put it with another pox virus, the heat-killed one will be reactivated. This seems a logical way to look for an insect pox virus, but so far results with this have been negative. We can't get the insect pox virus to reactivate any of the vertebrate pox viruses, nor can we go the other way. We find that you don't get interference with these viruses either. Using electron microscopy and bioassays, tissue cultures and supernatants and this type of thing, it seems apparent that the insect virus is recognized by the two vertebrate lines that we've tried. They are simply not taken up, the cells reject them, almost completely. Yet the virus is covered with a "halo," which goes into the inclusion. It is apparently made as the virion goes into the inclusion. This is a rather nondescript protein which the cell doesn't recognize and virions, although uncoated, get within the cell. Animal pox viruses occur in the aquatic environment, but they have not been tested very well at this point. The existence of this type of virus in insects should have no adverse bearing on the concept of using viruses in insect control.

WILLIAM UPHOLT: I hear much talk about "morphologically similar" viruses occurring in widely different organisms. I am disappointed there hasn't been more comment on the rate of mutation. What I'm not clear on is whether organisms with so similar morphological characteristics might be subject to mutation that might make them susceptible to or make them infective to quite different organisms. This seems to me to be a very important question.

IGNOFFO: It's very difficult to answer that question in terms of rate of mutations. We know these mutations exist in nature and of course we are exposed to many viruses, not only plant viruses, even in our everyday intake, and even tobacco viruses, of course, not necessarily through inhaled smoke but certainly through contact. We're continually exposed

to insect viruses and these things have occurred in nature for a long time, so whether one evolved into another form which eventually evolved into one which man or higher primates may be susceptible to is a difficult question to answer. We do have techniques, though, that will induce mutations. And there is a possibility of setting up an experimental program to induce mutations and then follow this up with testing to see if the host spectrum has changed. Accompanying this, you not only can induce for mutation and test for a host spectrum change, but also go through a process of continuing selection. In a group of 20 or 30 different forms, or what we might call a collection or an isolate, there might be forms that we can select for by mutation. Then, by actually exposing the mutagens or other types of mutating substances, we can select for those in each time test and see if the host spectrum has changed. We did do one type of test with NPV's. Actually, Dr. George Allen and I, in two separate laboratories, tried to do the reverse. Could we select for resistance in an NPV? Now it's the other side of the spectrum. We took it through 25 generations, under pretty heavy pressure, and at the 25th generation and all the way along, we had no difference from our non-selective strain of insects nor did we have any difference from wild populations tested. We have to ask ourselves if the question is meaningful, if it can be put to the experimental approach. And if it is, then we should do it.

J. E. ZAJIC: In one of Dr. Anthony's slides, I believe there was a polyhedral inclusion which was the example he was describing and then there looked to be some spherical inclusions above it. So my question is: Is it common to have two types of inclusions in the same system, do you encounter this very frequently?

ANTHONY: That was probably a dual infection of NPV and CPV. We also have seen dual infections of an NPV and a CPV in *Uranotaenia sappharina*. I don't think that this really occurs too often; however, in the particular epizootic Dr. Clark investigated, dual infections were quite common. In one pond near our laboratory, where Ed Hazard found the NPV and CPV in *Uranotaenia*, a number of the specimens examined also had dual infections. But I cannot give you any specific answer as to how often this occurs in nature.

IGNOFFO: A little enlargement on that question, with insects other than aquatic insects: it does occur, it's not uncommon to find them, but it's not the general picture. Those weren't both DNA viruses, were they?

ANTHONY: No. This is perhaps one thing that I should have brought out. All of the nuclear polyhedrosis viruses investigated are DNA viruses, while those cytoplasmic polyhedrosis viruses investigated are RNA viruses. Also, the so-called ICDV's, of which the iridescent viruses are a group, are all DNA viruses, whether they are from vertebrates or from insects or plants.

R. J. MIGET: There has been much discussion about various biological controls and it had to deal mostly with mosquitoes, it seems, as pests. Now, aside from the health hazard problem with mosquitoes, encephalitis or whatever, which we don't have now in this country, my question is: Has anyone looked at the ecological role that mosquitoes play? Is the thought to go out and spray the boondocks in south Louisiana, or to infect with a biological agent, or to infect, say, urban areas that have drainage problems? And this gets into how far mosquitoes can migrate. Do you want to wipe out the ones in the towns or in the country? If you wipe out the ones in the country by mass application, then what do you do to the ecology?

IGNOFFO: That's a good question, one to which people are addressing themselves. What will come in to fill the void of the ecological balance once it has been eliminated? I'll ask Dr. Chapman to answer it since he has the msot experience in this area.

CHAPMAN: That is a good question, we hear this quite a bit at Rotary Clubs and various things along this line. They ask, What good is a mosquito?--that's the next question. Then I tell them it's given me a pretty good livelihood for 25 years, my wife appreciates it. We are not attempting to eliminate mosquitoes, really. All we would like to do is to reduce them to a tolerable level, be it for man or animals down in our area. We haven't been able to eradicate mosquitoes by chemical means and we don't expect to eradicate them with biological means. We also would like to think that our attempts to reduce mosquito populations would be in areas where this would be necessary, in areas where we have shown we cannot reduce them by source reduction, and approaches like this. Too often we rush in to control mosquitoes and similar pests using a chemical or something along this line, where we could just as well empty the tin cans, drain the ditches, or use impounded areas. We have to be very careful that first we try to eliminate mosquitoes by all naturalistic means and then approach the problem with, hopefully, some sort of dual approach, maybe some sort of chemical and biological control agent.

FUNGAL PARASITES OF MOSQUITOES*

Donald W. Roberts**

INTRODUCTION

Entomogenous fungi cannot be offered at this moment as alternatives to currently employed methods of mosquito control. However, there have been some very promising discoveries concerning these fungi throughout the world in the last five years. Few people are working in this area, thus development has been slow. Enough information is available, however, to reveal that some fungi have considerable potential, both in terms of controlling mosquitoes and reducing synthetic pesticide pollution in the environment.

PATHOGENS

The discussion will be restricted to five fungal genera, ranging from small water molds, *Coelomomyces* (Blastocladiales) and *Lagenidium* (Lagenidiales), through higher Phycomycetes, *Entomophthora* (Entomophthorales), to Fungi Imperfecti, *Beauveria* and *Metarrhizium* (both Moniliales). Other fungal pathogens of mosquitoes have been reported, but these either show little promise for mosquito control or are insufficiently studied at present to be evaluated. The older literature has been covered (27). More recent publications treat *Pythium* sp. (7), *Coelomycidium* sp. (60), *Fusarium oxysporum* (24), and an unidentified Deuteromycete (61).

COELOMOMYCES

There are approximately 40 species of *Coelomomyces*, some of which are, as yet, undescribed.

Host Range.--All *Coelomomyces* spp. are aquatic insect pathogens, the majority of which infect mosquitoes. Other than one species reported from Notonectidae (3), their hosts are dipterous insects: Culicidae, including all major genera (e.g., *Anopheles*, *Aedes*, and *Culex*) (13), Psychodidae (33), Chironomidae (38, 48, 49, 69, 70, 71), Simulidae (19) and Tabanidae (2, 28). There is one report of the fungus in a non-insect

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host. Four specimens of a crustacean, *Daphnia*, collected along with *C. indiana*-infected *Anopheles subpictus* larvae, were found to contain a few sporangia of the same *Coelomomyces* (23) but it was not specified whether they were in the gut or hemocoel. Some species are reported from a rather wide range of mosquitoes, but the majority appear to be restricted to one or a few mosquito species.

Distribution.--*Coelomomyces*-infected insects have been collected from all continents except Antarctica. Distribution has been discussed (13, 27).

Life Cycle.--Irregular shaped hyphae without cell walls develop in the hemocoel of infected larvae. Sporangia are produced within the hyphae at their tips. Motile cells are produced and released from the sporangia. The process of motile cell differentiation and release is called dehiscence. The motile cells have been called "zoospores" in the literature, but since their function is unknown (they could be gametes, zygotes, or zoospores) it is more appropriate to refer to them as "planonts" (37). The role of these planonts in disease induction is unknown. Exposing larvae to suspensions of planonts alone has not resulted in rapid development of disease, even though this would be expected to occur if planonts were the infective unit of *Coelomomyces* spp. The release of planonts, however, is essential to disease development since in laboratory studies, sporangial preparations with low percentage dehiscence induced fewer infections than ones with high dehiscence levels (B. A. Federici and D. W. Roberts, unpublished). Pulse-label type experiments were conducted in which larvae were held for 6-day intervals in containers to which dehiscing sporangia had been added. When removed to clean water, larvae which had been exposed during the first 6 days did not develop infections, whereas those exposed during the second 6 days were infected (17). Since it is improbable that planonts could survive 6 days, these observations are interpreted as indicating *Coelomomyces* may have an as yet undescribed stage in its life cycle, and this stage itself is, or is the producer of, the infective units of the fungus.

Sporangial dehiscence of *C. psorophorae* from *Aedes taeniorhynchus* and *Psorophora howardii* was enhanced by exposing sporangia to homogenates of mosquitoes and certain amines and amino acids, including Tris [tris(hydroxymethyl)aminomethane], methionine, citrulline, and glycine (53). Indolebutyric acid somewhat stimulated dehiscence of *C. psorophorae* from a non-floodwater species, *Culiseta inornata* (42).

Infection.--Attempts to obtain infections in the laboratory seldom succeed. Exceptions include *C. punctatus* and *Anopheles quadrimaculatus* (10, 11, 17), *C. indicus* in *Anopheles gambiae* (36), *C. psorophorae* in *Culiseta inornata* (Chapman, personal communication; 17; H. Whisler, personal communication), *C. psorophorae* from *Aedes taeniorhynchus* (17), *C. tasmaniensis* (=opifexi) in *Aedes australis* (45), and *Coelomomyces* sp. in *Aedes atropalpus* (54). A common feature of all successful trials seems to be the presence of soil or algae.

Coelomomyces-infected larvae have been collected from all typical types of mosquito habitats, including lakes, ground pools, treeholes, tidal pools, husk pits, cesspools, discarded tires, and other discarded receptacles. Fresh, brackish, clear, muddy, and polluted water are involved (47). The latter is particularly interesting in view of the apparent fragility of the fungus.

Artificial Culture.--None of the *Coelomomyces* spp. have been cultured free of their hosts. Limited growth, which terminated after 36 hr, has been obtained with mycelium dissected aseptically from infected larvae and incubated in mosquito tissue-culture medium (M. S. Shapiro and D. W. Roberts, unpublished). Also, spherical pigmented bodies were produced on mycelium held for several weeks in association with mosquito tissue-culture cells, but separated from them by a membrane (Roberts, unpublished). If *Coelomomyces* spp. have a saprophytic phase which produces infective units (17), then artificial culture of this phase is theoretically more simply accomplished than culturing of the parasitic phase. It is possible, however, that the phase outside mosquitoes may develop on some other organism, such as algae.

Microbial Control.--*Coelomomyces* has several attributes which make it promising as a control agent for mosquitoes, either with or without the help of man. First, although the level of disease in most populations is low (less than 10% infected), very high levels have been noted in some instances (5, 63), e.g., 95% in *Culiseta inornata*, 95% in *Psorophora howardii*, 97% in *Aedes triseriatus*, and 85% in *Culex peccator* (H. C. Chapman, personal communication). Second, current information indicates considerably more host specificity than that present in available chemical pesticides. Third, there are four examples of successful introductions into new sites. In one of these, the Tokelau Islands, a fungus from one mosquito species, *Aedes aldopictus*, was successfully colonized in another species, *Aedes polynesiensis*, and remained active in the new locality for at least seven years (31). The other three examples involved no change in host. *Anopheles gambiae* larvae were infected in a previously disease-free pool three weeks after introduction of sporangia (41). Mortality approached 100% in later generations of mosquitoes. Introductions of sporangia and larvae into artificial pools in North Carolina produced an average of 60% infected larvae (11). Prior to drying at the end of the growing season, a rice field in Egypt was treated with sporangia from *Anopheles pharoensis* (18). Larvae were collected from July to September the following season and 94% of those collected in August were found to be infected. Three other fields were treated early in the season. In two of these, infection levels reached 90% in early August and then receded to 0%. The third field had no infected larvae.

The third field illustrates one of the current disadvantages of *Coelomomyces* as agents for mosquito control, viz. their performance is unpredictable. This situation presumably stems from the superficiality of our knowledge concerning the group. Perhaps the most serious lack of information is our failure, more than 50 years after the fungi were

first found, to elucidate their life cycle(s). Uncertainties concerning the infective unit, site of infection, and appropriate chemical environment for disease induction, make predictable results in laboratory and field infection studies virtually unobtainable. Many lines of investigation are impeded at present by our inability to culture the fungus in vitro. Although field observations indicate *Coelomomyces* are safe for vertebrates and most invertebrates, documentation by laboratory experiments is needed. This can be accomplished best after the infective unit has been identified and can be produced aseptically in large amounts in artificial culture.

LAGENIDIUM

Lagenidium giganteum was described in 1935 (8), but for many years it was thought to hold little promise for mosquito control (9). One isolate which was obtained from culicine larvae in 1969 in North Carolina, however, has demonstrated considerable potential for use in mosquito control (35). Although references have been made to it as *L. culicidum* (34, 64), it is properly called *L. giganteum* (12).

Host Range.--The original description of *L. giganteum* was based on material from copepods, *Daphnia*, and mosquito larvae. Umphlett's isolate, however, in a series of laboratory tests has infected only mosquitoes (35). Late fourth instar larvae, pupae, and adults were not susceptible, whereas early fourth instar and younger larvae succumbed. The non-mosquito invertebrate organisms exposed to zoospores included *Cyclops*, *Daphnia*, *Scapholeberis*, several additional unidentified species of copepods and cladocerans, crayfish, polychaetes, dytiscids, chironomids, and snails. The vertebrates tested were fish (*Lebistes* and *Gambusia*), birds (chickens and quail), and rodents (rats). The mosquito species tested were *Aedes aegypti*, *Aedes triseriatus*, *Aedes mediovittatus*, *Aedes taeniorhynchus*, *Aedes sollicitans*, *C. pipiens quinquefasciatus*, *C. fatigans*, *C. tarsalis*, *C. nigripalpus*, *Anopheles albimanus*, *Anopheles quadrimaculatus*, *Anopheles stephensi*, and *Anopheles sundaicus*. All except the anophelines were susceptible (34). Higher levels of inoculum resulted in some infection of *Anopheles quadrimaculatus*, but the 100% mortality routinely obtained with culicine species was not attained (65; P. Giebel and A. A. Domnas, personal communication). Field studies with *C. restuans*, *C. tarsalis*, *Aedes nigromaculis*, and *Psorophora* sp. indicate these species are highly susceptible (35, 65). Nine isolates other than Umphlett's have been obtained in Couch's laboratory (12).

Distribution.--Mosquito-parasitizing isolates of *L. giganteum* have been collected in North Carolina and India (8, 12, 64, 66). The fungus was collected in England using termite wings as bait, but this isolate was not tested for pathogenicity to mosquitoes (72).

Microbial Control.--The attractive features of *L. giganteum* include its wide spectrum of host mosquitoes, rapid rate and high level of lethality, ease of production on artificial media, and its ability to persist in mosquito habitats. The latter is implied by Umphlett's isolation of *L. giganteum* from the same site after a 6-year interval. Disadvantages include its virtual restriction, at least at reasonable dosages, to culicine mosquitoes (it is possible, however, that isolates other than Umphlett's will be better adapted to attacking anophelines); lack of tolerance for NaCl and organic pollutants; short-lived sporangia and zoospores which make storage for more than a few days impractical; and the incomplete evaluation of the safety of the organism for nontarget organisms. The latter is particularly true for the isolates originally obtained from non-mosquito hosts.

Two field tests have been conducted, both of which gave promising results. *Culex restuans* disappeared from an artificial pool in North Carolina 6 days after introducing 120 *Lagenidium*-infected larvae (65). An untreated control pool was not included in this test but the fact that 43% of the *C. restuans* collected 3 days after initiation of the experiments were infected indicates the drop in population was due to *L. giganteum*.

The second trials were conducted in California in the late summer of 1972 (35) with two insecticide-resistant mosquitoes, *Aedes nigromaculis* and *C. tarsalis*. The population of the former was declining as the test was initiated, but introduction of the sporangia produced in one infected larva (a potential of approximately 250,000 zoospores) per square foot of water surface greatly increased the rate of decline over untreated sites. After 3 days, 75 larvae were collected in the single control site and 0-3 larvae in the three test sites. With *C. tarsalis*, the population was more stable and sampling was continued longer. Three sites were chosen, one high in dissolved solids at pH 10, one with conditions resembling that of rice fields at pH 8, and one with high chloride ion levels. Infections occurred in the first and last sites, but after an initial wave of up to 25% infected 3 days after introduction of the fungus, the levels dropped to 1-9%. In sites of the second type, numbers of *C. tarsalis* collected gradually dropped to zero over a period of 5 days. The population was still zero, as compared with 111 larvae in control sites, 17 days after fungus introduction.

The fungus presumably survives periods of adverse conditions as zygotes produced by fusion of antheridial and oogonial nuclei. Germination of this relatively heavily walled body has not been observed in the laboratory. Elucidation of the factors which induce their production and germination would make available a form of *L. giganteum* which could be shipped and stored for use in mosquito control tests. Cadavers, if stored in fresh tap water at 15.6 C (60 F) before sporangia began forming zoospores, remained useful for induction of disease for up to 14 days (34). If cadavers are to be used in extensive field tests, considerable improvement will be needed in methods of storage.

ENTOMOPHTHORA

Entomophthora infections take place primarily in adult rather than larval mosquitoes. The only exception is *E. aquatica*, which has been collected only in Connecticut (1). The levels of infection, particularly in overwintering populations of adults, frequently approach 100% and epizootics have been noted in some sites over periods of several years.

Host Range.--*Entomophthora aquatica* was found in infected larvae and pupae of *Aedes canadensis* and *Culiseta morsitans* (1). Attempts to infect larvae in the laboratory failed. Attempts to infect *C. pipiens* with an *Entomophthora* sp. from *C. pipiens* using field-collected infected adults as inoculum gave the following results: third instar and younger larvae = 0% infected, fourth instar larvae = 25%, pupae = 63-88%, male adults = 33-67%, and female adults = 65-100% (22). Adults of *Anopheles maculipennis messeae*, *Anopheles maculipennis atroparvus*, *Aedes dorsalis* and *Aedes aegypti* were resistant to this fungus. The several reports of either *E. conglomerata*, *E. destruens*, or *Entomophthora* sp. infecting *C. pipiens* in nature indicate the fungal isolates involved may be somewhat species specific. *Culiseta annulata* adults were found less susceptible than *C. pipiens* collected in the same overwintering sites (4, 43).

Distribution.--*Entomophthora destruens* occurs in Czechoslovakia, France, and England (68) in cool (1-20 C), moist (44-100% R.H.) *C. pipiens* resting sites all seasons of the year, but was most prevalent in winter months (44). The *Entomophthora* sp. in the Netherlands also occurred in overwintering adult populations (62). On the other hand, *Entomophthora* sp. and *E. conglomerata* infecting *C. pipiens* adults associated with sewage disposal and water filtration systems have been found in the USSR in the summer months (20, 26, 30). *E. aquatica*-infected larvae were collected from pools near a single stream in Connecticut during the summer months (1). The levels of infection varied extensively (0.8-80%) from site to site.

Microbial Control.--The high levels of infection noted in nature indicate *Entomophthora* spp. hold considerable promise for control of adult mosquitoes, particularly *C. pipiens*. Nevertheless, artificial introductions of these fungi into new mosquito breeding or overwintering sites apparently have not been attempted. In addition to causing high mortality, these fungi are attractive because they can remain active in a site year after year (44). It is possible that a single introduction would be adequate to establish a fungus for many years.

Production methods, particularly production and germination of resting spores, need improvement. Conidia are short-lived. Infected adults were no longer useful as inoculum 8 days after the first conidia were produced (22). *E. destruens* has been cultured on coagulated egg yolk and other media, but there is no mention of the infectivity of this material (68). *E. aquatica* could be cultured only on coagulated egg yolk, but grew abnormally (1). Neither cultures nor infected field-collected

specimens induced infection in the laboratory.

The safety of *Entomophthora* spp. for vertebrates and other non-target organisms needs verification. Aside from *C. coronatus*, no infections of vertebrates have been reported for insect-parasitizing Entomophthorales; but experimental exposure in the laboratory has been carried out only with resting spores of *E. thaxteriana* from aphids (R. Soper and F. Holbrook, personal communication). The observation that *E. destruens* and *Entomophthora* sp. from *C. pipiens* did not attack other mosquito species (21, 43) indicates these fungi are more host specific than *C. coronatus*.

Infected adults have been collected in overwintering sites, in sewage systems, in water treatment systems, and on vegetation surrounding reservoirs. These sites would appear to be the most promising types of mosquito habitats for artificial introduction of *Entomophthora* spp. Such introductions, however, should be preceded by basic research on the requirements of the fungus to be introduced. For example, even though *E. destruens* induced 100% mortality of *C. pipiens* in two overwintering sites, it was not effective in similar sites which had been painted recently. Also, it was effective on brick but not on plaster (43, 44).

BEAUVERIA

Various isolates of *Beauveria bassiana* and *B. tenella* (*B. brongniartii* [14]) infect a wide spectrum of insects, but there is little information concerning their effects on mosquitoes (27). Only one epizootic in natural populations of mosquitoes had been reported, viz. *B. tenella* in larvae of the treehole mosquito, *Aedes sierrensis* (46, 57), near San Francisco, California.

Host Range.--An isolate of *B. bassiana* obtained from a non-mosquito host was tested for virulence against larvae of *Anopheles albimanus*, *C. pipiens*, *C. tarsalis*, *Aedes aegypti*, and *Aedes sierrensis* (6). The first three species were infected, but both *Aedes* species were not. Adults of the same five species, plus *Aedes nigromaculis*, were all highly susceptible to conidia applied directly to the insects or to environments into which adults emerged from pupae. *B. tenella* isolated from larval *Aedes sierrensis* was tested against other species using blastospores produced in submerged cultures (46). The rate of kill was slowest with the native host, *Aedes sierrensis*.

Microbial Control.--Three small-scale outdoor tests with conidia of *B. bassiana* caused 82, 95, and 69% reductions of *C. pipiens* larvae and pupae present after 2 weeks (6). The dosage used was equivalent to 3 lbs/acre. Treeholes treated with 5×10^3 or 5×10^5 blastospores of *B. tenella* per milliliter of water had reductions of 53 and 71%, respectively, in production of *Aedes sierrensis* adults (46). Fifteen treeholes were treated with each dosage level and 15 served as untreated controls. Up to 92% of larvae collected from some treeholes

in which natural infection occurred were found to be diseased. Readings of treated treeholes were terminated after 72 days, but it is possible that the fungus will remain active in the treated sites in future seasons. Field tests of this fungus against more susceptible mosquito species may show greater population reductions.

As mentioned previously, adults of all species tested were highly susceptible to *B. bassiana* conidia. Since mosquito adults tend to rest in habitats favorable to fungal infection, treatment of such sites with conidia has been suggested as a promising area for research (6). *Aedes nigromaculis* adults, which rest on grass after emerging and between blood meals, have been mentioned as a species particularly amenable to treatment as adults.

Mass production of conidia is a relatively simple matter since the fungus can be grown on a wide variety of artificial media, including sterilized vegetable matter such as bran (39, 40, 58). Conidia cannot be produced in liquid culture with present technology, but production is possible in aerated semisolid media (E. Westall, personal communication). Blastospores, on the other hand, are produced only in liquid media (55, 56) and presumably could be produced using existing large-scale fermentation technology. Boverin, a mixture of *B. bassiana* conidia and mycelium which is produced in Russia for control of leaf-feeding insects, had little effect on mosquito larvae in a laboratory test (15).

Larvae were susceptible to *B. tenella* blastospores at any time, but were susceptible to floating *B. bassiana* conidia only during the first 24 hours following molts. In addition, *Aedes*, a large and important genus, was not susceptible to conidia as larvae; and conidia had no residual effect because they germinated in mosquito habitats even when not in contact with larvae. These limitations, along with the high dosages needed, led to the conclusion that the use of *B. bassiana* conidia for larval control is not promising (6). Nevertheless, from the results of preliminary experiments formulation with oil may overcome some of the problems.

The most serious problem concerning *Beauveria* at present is a question concerning its safety to vertebrates. Sensitivity has been reported by persons exposed repeatedly to massive amounts of *B. bassiana* conidia preparations (actually finely milled whole cultures grown on vegetable matter) (25). The active agent, whether a toxin or antigen, was not isolated. The sensitivity, however, could be overcome by the simple expedient of using long-sleeved shirts, gloves, and respirators while handling the preparations. Long-term rodent tests with *B. bassiana* conidia proved negative (C. Rehnborg and E. Westall, personal communication). These tests included inhalation, subcutaneous injection, and oral toxicity. Sensitivity to blastospores has not been reported.

Host range as far as nontarget aquatic invertebrates and vertebrates also needs close scrutiny. *Beauveria* is one of the most frequently isolated entomogenous genera, has cosmopolitan distribution, and an extremely wide insect host range. Isolates with minimal effects

on nontarget insects should be sought for field studies.

METARRHIZIUM

Most isolates of *Metarrhizium* are identified as *M. anisopliae* or *M. brunneum*. *M. brunneum*, however, is probably a brown-spored form of the green-spored species *M. anisopliae* (32, 67; M. G. Tulloch, personal communication). Although *M. anisopliae* has a very wide insect host range (200 species, primarily subterranean coleopterous larvae), it has not been isolated in nature from aquatic insects (67). Nevertheless, mosquito larvae proved very susceptible to conidia of one isolate (F84-1-1) in laboratory tests and preliminary outdoor trials (50, 51; F. Murphey and D. W. Roberts, unpublished). The conidia of this species are exceptionally hydrophobic and, unless treated with a wetting agent, float on the surface of water for days or weeks. Except where specified otherwise, the spores used in tests discussed below were not treated, and therefore were floating.

Host Range.--The species tested included *Anopheles stephensi*, *Anopheles quadimaculatus*, *Anopheles albimanus*, *Aedes aegypti*, *Aedes atropalpus*, *Aedes taeniorhynchus*, *Aedes sollicitans*, *Culiseta inornata*, *Culex pipiens pipiens* (4 strains), and *Culex restuans* (51). All were susceptible, but *Aedes aegypti* was susceptible to submerged spores only.

Some field-collected aquatic organisms were susceptible to conidia in the laboratory (Roberts, unpublished). Interpretation of these experiments is uncertain because of high mortalities of some untreated controls. Adult gyrinid beetles and broad-shouldered water striders which live at the surface of the water were very susceptible and slightly susceptible, respectively. Damselfly and mayfly naiads were somewhat susceptible and dragonfly naiads, amphipods and snails were not susceptible. Guppies exposed to massive amounts of conidia for two months were not affected.

Microbial Control.--In laboratory tests the F84-1-1 wild type of *M. anisopliae* routinely induced 98 to 100% mortality in larvae of several mosquito species. Populations of late instar *C. pipiens pipiens* larvae were severely reduced in outdoor tests conducted in 1972 using small containers (0.25 meter^2 surface, ~20 liters of water). More recent tests with 300 and 600 mg of conidia/m^2 in small artificial ponds reduced *C. pipiens pipiens* by 91% and 94% within 3 days and with *Aedes sollicitans* by 85% and 98% (F. Murphey and D. W. Roberts, unpublished).

All tests were conducted with pure conidia. It is presumed that formulations could be devised which would permit reductions in dosage. Since inactivation of conidia by direct sunlight has been noted in laboratory studies, if possible, formulation should include compounds to protect against ultraviolet irradiation.

Mass production of *M. anisopliae* conidia on artificial media was

first reported in the late 1880's (29), and has been successfully accomplished by a number of researchers (39). The fungus will grow on very simple artificial media, including ones such as Czapek-Dox medium which contains no organic nitrogen. For mass production, however, as a matter of simplicity, sterile vegetable materials (usually bran or rice kernels) are commonly used. The medium on which the conidia are produced affects their virulence. In comparisons between conidia produced on a starch medium with yeast extract (YpSs [16]), a peptone medium with yeast extract (SDAY), and an inorganic nitrogen medium (CDB), the YpSs-produced spores consistently killed larvae approximately 40% more quickly than spores from the other two media (Roberts, unpublished). Spores from YpSs also had the highest initial viability and retained viability longest on storage. This, however, did not account for the increased rate of kill since viabilities of spores from the different media were equalized prior to testing by addition of appropriate amounts of autoclaved spores to the two lots with higher viabilities.

As with the other fungi, safety data are meager. *M. anisopliae* is a cosmopolitan species, but no infections of warm-blooded animals have been reported. Substituting filtrates for water for two weeks had no discernible effect on young mice (Roberts, unpublished), and long-term tests with rodents indicated no adverse effects (C. Rehnborg and E. Westall, personal communication). *M. anisopliae* is neither toxic nor pathogenic to rats (59). Rats fed a mixture of fungus-contaminated and uncontaminated food lost weight, and a few of the specimens fed only contaminated food died. It is possible that these effects resulted from severe depletion in nutritional value of the food by extensive fungal growth. There are no reports of human sensitivity to *M. anisopliae*.

Since conidia are not produced on fungus-killed mosquito larvae, *M. anisopliae* will be used in mosquito control programs as synthetic insecticides are now employed, viz. introduced repeatedly, rather than being colonized in new sites through single introductions. Since production on simple artificial medium is possible, this organism could be useful as a local product for nations desiring to conserve foreign capital.

CONCLUSIONS

It is apparent that there is insufficient information to assure predictable results with entomogenous fungi for mosquito control. Types of information needed are the same as those specified for fungi used against all types of insects (52).

The fungi known to infect mosquitoes offer a broad range of possibilities for use in mosquito control (Table 1). Organisms amenable to colonization into new sites include *Coelomomyces* spp., *Lagenidium giganteum*, *Entomophthora* spp., and *Beauveria tenella*. *Metarrhizium anisopliae* is best suited for use as synthetic pesticides are now applied, viz. introduced in large amounts each time the mosquito population

becomes excessively large. In addition to fungi known to be pathogenic to mosquitoes, it is possible that fungi with good potential for mosquito control will be discovered (or rediscovered) in the future. Two recent examples of rediscovery are the observations that certain isolates of *L. giganteum* (65) and *M. anisopliae* (50, 51) are extremely virulent for mosquito larvae. The most recent report of a new discovery is an unidentified imperfect fungus in Australia (61).

TABLE 1. Possible Uses for Selected Entomogenous Fungi in Mosquito Control

Fungus	Mosquito stage	Mosquito genus	Habitat
Coelomomyces spp.	Larvae	All major genera	Aquatic, including brackish water with some *Coelomomyces* species
Lagenidium giganteum	Larvae	Culicine genera	Aquatic, not brackish nor high organic pollution
Entomophthora aquatica	Larvae, pupae	*Aedes*, *Culiseta*	Aquatic
Entomophthora spp. and *E. conglomerata*	Adults	*Culex*	Water surface and vegetation surrounding water works and sewage systems
E. destruens	Adults	*Culex*	Overwintering and dark, moist resting sites
Beauveria tenella	Larvae	All major genera	Aquatic, including treeholes
B. bassiana	Adults	All major genera	Resting sites of adults
Metarrhizium anisopliae	Larvae	All major genera	Aquatic, including brackish and possibly organically polluted water

Entomogenous fungi, without the assistance of man, have been found affecting substantial proportions of some mosquito populations. This suggests that manipulation, such as modification of sites or increasing inoculum potential, would make fungi more efficient in mosquito control. While success in such ventures can be expected, they must await the availability of basic information to guide us in making intelligent use of the strengths and recognizing the limitations of each fungus.

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DISCUSSION

E. M. McCRAY: Prior to 1969 our efforts at the Center for Disease Control in control of vector-borne diseases had been directed primarily toward use of conventional pesticides to control the primary vectors of malaria, yellow fever, filariasis, encephalitis and Chagas' disease. Some work was being done on genetic manipulation, nuclear radiation, and chemo-sterilization. To a lesser extent, the potential of pathogens and predators was being evaluated. We were not seeking new organisms, per se, but were looking at what was being done by other laboratories. At that time, most of the microorganisms lacked certain qualities that we were interested in. The aquatic fungi appeared to be the group which would most nearly fit our needs. We talked with two men--Dr. Couch, at Chapel Hill, who was working on *Coelomomyces*, and Dr. Roberts, at Boyce Thompson Institute, who was working at that time with *Metarrhizium*. We fully expected one or both organisms to be ready for field trials within a few years. The isolation of *Lagenidium* in the fall of 1969 by Dr. Clyde Umphlett was totally unexpected. Dr. Roberts, I think, has covered the life cycle very well, so what is the current status of our investigations?

In January of this year, 3 culex larvae were sent to us from our station in El Salvador for examination. These larvae were infected with *Lagenidium*, probably *L. giganteum*. This is the first report of this particular organism in that area. Unfortunately, the larvae were preserved in Bouin's fixative and were of no value in establishing a fungal culture. The investigators returned to the original site to seek additional infected larvae, but it no longer existed. Adjacent streams will be examined in the future.

Work at our laboratory is primarily on the dormant or resting spore of this organism to develop methods for accumulating and storing large quantities of the infective material. Dr. Umphlett, now at Clemson University, is working on a practical method of in vitro culture of the organism. Dr. Domnas, at Chapel Hill, is working on biochemical relationships between host and parasite. One small field test was started summer before last in an area outside Savannah, Georgia. The local mosquito control commission had a *Culex* breeding area that had been a continuous problem for them for about 12 years. We introduced spores of *Lagenidium* to see if we could reduce the natural population. For the past 2 years we've been unable to find a single mosquito larva in this site, which we would like to think was the result of our fungus.

What about the effect of this fungus on non-target organisms in the environment? The literature reports that the Lagenidiales and many of the other aquatic fungi are parasites of a very large number of aquatic organisms, many considered vital in the food chain. Obviously, a given organism cannot possibly be screened against every single species in the environment. Two avenues of approach are readily apparent.

One is to bring selected non-target organisms into the laboratory for screening; the second is to take the organism into the field in a carefully controlled environment and see what happens. The latter approach will be necessary sooner or later. Hopefully, some host parameters will be defined in the laboratory or by natural occurrences prior to field introduction.

There are two problems which we in the public health service feel more research is urgently needed for: (1) The development of a reasonable set of guidelines for conducting laboratory tests with pathogens on non-target organisms. We have procedures for chemical pesticides, but not for pathogens. Unfortunately, pathogens are not chemical compounds which behave in certain clearly defined ways. Each organism will, of necessity, require unique approaches, but at least we should be able to come up with some basic procedure or scheme which will enable us to relate comparable tests. We in the public health service want from those outside the public health service to offer us such guides. (2) What is the natural occurrence and distribution of the more promising organisms and what are the environmental factors which determine their presence or absence in any given site or area? Within the past few weeks, an agreement has been reached between CDC and Dr. Washino at the University of California at Davis, to conduct intensive laboratory studies on the possible effect of *Lagenidium giganteum* on selected non-target organisms characteristic of rice fields. With this information, we should be able to reduce the unfavorable impact on the environment.

D. T. GIBSON: Most of the safety tests that have been suggested today have been directed toward infection of non-target organisms. Are toxins produced in the target organism? As well as spores, perhaps infected mosquitoes also should have been given to the guppies to see if there was any toxic effect.

ROBERTS: Some fungi are well known for their toxins, e.g. *Aspergillus flavus* and aflatoxins. This is an area we must examine in evaluating fungi for insect control. There are several problems.

If a fungus makes toxins in vitro does this mean it is to be rejected out of hand for insect control? Or is the way the fungus will be used to be taken into consideration? For example, the characteristics of some entomogenous fungi are such that in insect habitats, unlike artificial media where there are no other microbial competitors for nutrients, there is virtually no saprophytic growth and, consequently, no toxin production. As mentioned by Dr. Gibson, safety tests should include determination of whether amounts of toxins present in infected hosts are sufficient to be deleterious to predators or scavengers. The artificially disseminated units usually are spores. Do these spores contain significant amounts of toxins? If not, and if the medium on which the spores were produced is to be discarded, then toxins produced in vitro are of little importance since they will be discarded with the culture medium. I think all of us who work with fungi realize we must solve these types of problems. *Lagenidium* and *Coelomomyces* being lower fungi, a group not known to produce toxins, probably will require less stringent examination for problems from toxins than will the higher fungi.

MARSHALL LAIRD: Dr. Roberts' point is well taken about the obvious need to understand the mechanism of infection in *Coelomomyces* as a basis for studying effect on non-target organisms, but perhaps there's another aspect of safety that one shouldn't lose sight of. For example, workers using DDT in the old days were supposed to wear face masks, and I've seen teams working under extremely hot conditions in Pakistan where the man applying the chemical or mixing it up had a very large beard and a turban. Wearing a face mask was grossly uncomfortable and if the boss wasn't watching, he wouldn't wear it. One day, when the same gentleman or a successor is handling 100-lb sacks of *Coelomomyces* sporangia, one wonders whether he'll be at risk in terms of inhalation and possible lung infection on the same basis.

S. R. DUTKY: I have tested *Metarrhizium anisopliae* and *Beauveria bassiana* against housefly and *Aedes aegypti* adults, and both are very highly susceptible to these fungi. A second point of information is in reference to *Entomophthora coronata*, now known as *Conidiobolus coronatus*. We are studying the sterols of two fungi, *C. coronatus* and *E. apiculata*. These two have identically the same sterols. Cholesterol is the major sterol, and I don't know of any other case where this is true with fungi, except maybe one in South Africa. It would be unusual for two unrelated fungi to have the same sterol composition. I wondered if you were going to throw *E. apiculata* out, too.

IGNOFFO: May I address myself to that? I think each organism that shows potential, and this is going to vary from time to time as we decide which ones are the best, will have to be examined as to whether they are safe using the experimental approach. We are not necessarily restricted just to use of spores. In some instances, we're going to have to test possible metabolites produced in the host once infection takes place. I think what should be done is put the idea to the scientific method to determine its potential hazards and monitor it if necessary to determine if it will retain its original specificity. Because some materials have wide ranges of specificity does not necessarily exclude them from consideration as potential microbial insecticides. It makes us aware of a possible problem area, but that should be corroborated or, in fact, refuted by direct experimentation.

JOHN BRIGGS: This is a question to Dr. Singer. We have a euphemism in the western world for toxins from bacteria and fungi, and the word is "antibiotics." There is a great need to be concerned about the sensitivity not only to particulate materials like sporangia of *Coelomomyces* but also to the products of these materials. We are all aware that the risk-benefit ratio we exercise when we submit ourselves to antibiotic treatment is a risk-benefit ratio because some individuals have a sensitivity to metabolic products of fungi and bacteria. Since we are talking about impact on aquatic environments, aquatic environments sometimes carry metabolites to places other than where they were applied. When such substrates are removed, they are transferred to sites where non-target organisms may exist that were not considered initially. I was wondering, Dr. Singer, as a microbiologist, is there substantial evidence for degradation of metabolic products of bacteria and fungi? For example, is there degradation of antibiotics, as we know them, through sewer systems and others of this sort?

SINGER: You have to be very careful when you speak of antibiosis, the effect of one living entity on another, and antibiotics as delivered by various industrial outfits. The earth's soil is the great degrader, and most things will disappear like we will, fortunately (or unfortunately). When we speak about toxins we have to be specific. When I write on the *sphaericus* "toxin," I put it in quotes--I don't want to call it an endotoxin, because that confuses it with *Salmonella* and so forth. We have to be careful how we use words like ecology, toxin and antibiotic.

ALLEN LASKIN: I take issue with your euphemism, that toxins from bacteria and fungi are antibiotics. Antibiotic has a very specific definition. If I remember correctly, Dr. Waksman's original definition was that "an antibiotic is a substance produced by one microorganism which inhibits the growth of other microorganisms in very low doses." I think we can clearly differentiate toxins from antibiotics in that context.

THE USE OF MICROSPORIDA (PROTOZOA) FOR THE CONTROL OF AQUATIC INSECT PESTS

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ABSTRACT

The most important and promising protozoan diseases of aquatic insect pests are members of the Microsporida (Sporozoa: Protozoa). These diseases result in high mortalities in host larvae and cause noticeable reductions in the fecundity and longevity of individuals which survive to sexual maturity. Several species, evaluated in laboratory studies, show promise for control of mosquitoes, and others have been described which may be useful in control of black flies, chironomids, and semi-aquatic tabanids. Hundreds of additional species undoubtedly will be found in these insect pests as research programs are expanded to meet the increasing demands of environmentalists to replace chemical insecticides. A *Nosema* disease of anopheline mosquitoes, the subject of concentrated research by the U.S. Department of Agriculture for several years, will be tested against field populations of *Anopheles albimanus* Wiedemann this year in the Canal Zone. The research on this *Nosema* not only has demonstrated its potential use for the control of anopheline mosquitoes, but has also shown that it is harmless to all nontarget organisms tested including a local species of crayfish, fishes of the genus *Gambusia*, fresh-water shrimp, and several aquatic entomophagous insects. Microsporida (Sporozoa: Protozoa) have been reported in pest species of the families Ceratopogonidae, Culicidae, Simuliidae, and Tabanidae (a family that includes both aquatic and semi-aquatic species). Indeed more than 100 species of mosquitoes alone are known hosts of Microsporida. Several thorough reviews of these diseases in medically important insects are soon to be published, some of which reviews will establish new genera for morphologically distinct types found only in certain aquatic animal groups. For the present, the reader may consult Chapman (3) and Weiser (15, 16).

PROMISING SPECIES

In 1966, the U.S. Department of Agriculture, ARS, Insects Affecting Man Research Laboratory in Lake Charles, Louisiana, began intensive surveys of aquatic insect pests for microsporidan diseases. Many

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aquatic insect species have been found to be hosts of these enzootic diseases, and nearly all species of mosquitoes examined are hosts of one or more microsporidans. Many of these, particularly species of the genus *Thelohania*, were found to cause congenital diseases, but none appeared to be contagious or transmitted per os. Few species, therefore, were considered as promising biological control agents for aquatic insect pests. Recent studies have brought attention to development of these microsporidans in surviving females that pass the disease to their progeny via the egg. In these instances, *Thelohania* produce spores in adult females unlike those found in male larvae killed by the disease. These *Thelohania* would be useful control agents because they are all carried via the egg and produce up to 50% mortality in the progeny of infected females (10). In some *Thelohania* species, spores from adult females appear to be binucleate and may be infectious to larvae that ingest them; spores in larvae are uninucleate and probably never produce new infections in healthy larvae. All previous attempts to infect healthy insects by using spores from larvae have failed to produce disease symptoms. However, no serious attempts have been made to infect healthy mosquitoes by feeding them spores from adult females.

In contrast, the survey of aquatic insect pests in the tropical regions of Africa and Central and South America have recently uncovered many microsporidans that are transmitted per os. Perhaps some microsporidans have not developed transovarian sequences in tropical hosts, as is commonly seen in hosts in temperate regions, since many of these tropical hosts breed continuously throughout the year. To date, most of our research on tropical forms has been concerned with the taxonomy and distribution of these parasites, descriptions of which are soon to be published.

In addition to these new tropical microsporidans, we have six other species in aquatic insect pests that soon can be evaluated in field tests, two species in tabanids and four species in culicids. The two microsporidans in tabanids are (a) *Thelohania tabani* (6) from larvae of *Tabanus atratus* Fabricius and (b) a new undescribed species discovered by Dr. Donald Harlan and his colleagues at Stoneville, Mississippi (personal communication). Tabanids readily became infected, resulting in high mortalities when fed other diseased larvae in laboratory studies; however, a means of distributing these microsporidans in tabanid larval ecosystems must be investigated before they can be evaluated in field tests.

Four microsporidans that offer promise for control of mosquitoes are: *Nosema algerae* (12) in anopheline mosquitoes; *Pleistophora culicis* (14) in both *Anopheles* and *Culex* mosquitoes; a *Stempellia* species (soon to be described) in *Culex pipiens quinquefasciatus* Say; and another undescribed species of *Stempellia*, found in larvae of *Uranotaenia sapphirina* (Osteen-Sacken) in Florida and Louisiana (4), that also causes pathologies in the larvae of *Aedes aegypti* (L.).

The pathologies produced by *N. algerae* in anophelines have been described (2, 5, 7, 8, 12). High mortality has occurred in most *Anopheles*

species exposed to spores in laboratory colonies and in small experimental test plots. Perhaps more important is the ability of *N. algerae* to reduce longevity of adult mosquitoes that survive from less severe infections (5). Also, malarial organisms do not develop well in females infected with this *Nosema* (9, 13). Our laboratory is, therefore, conducting field evaluation studies with *N. algerae* against *Anopheles albimanus* Wiedemann, a primary vector of malaria in the Canal Zone. Large test plots (approximately 1.2 ha [0.5 acre]) will be sprayed with 1×10^6 spores/900 cm^2 [1 ft^2]). This concentration of spores usually produces 10-20% infection in larvae in the laboratory. Smaller plots will be sprayed with 2×10^7 and 2×10^8 spores/900 cm^2 to determine whether higher rates of infection and mortality can be produced. Data from these tests should be available in the summer of 1974.

Canning (1) studied the effects of *P. culicis* on *Anopheles gambiae* Giles in laboratory colonies and Reynolds (11) introduced the microsporidan into field populations of *Culex pipiens fatigans* Wiedemann on an island in the Pacific. Reynolds found infections in *C. p. fatigans* populations two years after the breeding areas had been inoculated with spores, but unfortunately the rate of infection was very low.

The *Stempellia* from *C. p. quinquefasciatus* causes low levels of infection in larvae. However, this microsporidan has a complex life cycle that results in production of two types of spores. One is produced in low numbers and is believed to initiate infections in larvae, while another is produced in large numbers which may not be infectious. The *Stempellia* found in larvae of *U. sapphirina* caused serious pathologies in *A. aegypti* in experimental laboratory tests and is also highly pathogenic to its natural host. However, additional experimental data are needed before the potential of this microsporidan can be properly evaluated.

Additionally, two *Pleistophora* were found in two species of *Culicoides* (4), but nothing is presently known about their mode of transmission. Also, many microsporidans are known from simulid larvae; however most are apparently transmitted via the ovaries. None of the described species have been shown to be infectious to healthy black flies in experimental studies.

HOST SPECIFICITY

During the last two years, we have concentrated our studies on diseases of all invertebrates and some fishes that live in the breeding areas of aquatic insect pests. These studies were deemed necessary to determine the host specificity of microsporidans under natural conditions. We have examined 116 species of aquatic animals, 60 insect pest species and 56 nonpest species, and have found a total of 122 microsporidan species. The latter represent species of the genera *Glugea*, *Mrazekia*, *Nosema*, *Parathelohania*, *Pleistophora*, *Stempellia*, *Telomyxa*, *Thelohania* and several undescribed groups (Table 1). No examples were

found where pest species shared microsporidan parasites with nonpest species and in only one case did species from different animal classes share the same parasite. A *Nosema*, a common parasite of amphipods, was found once in a planarian commonly associated with these crustaceans. All infected animals collected for these studies were prepared for electron microscopy, and the microsporidans examined in electron micrographs, using diagnostic ultrastructural characters to identify the species.

TABLE 1. Taxa of Aquatic Animals in Florida and Louisiana with Species that Are Hosts of Microsporidans

Taxon	Number of Hosts	Number of Microsporidans
Crustacea		
Amphipoda	8	11
Cladocera	5	5
Copepoda	5	5
Ostracoda	2	2
Notostraca	1	1
Oligochaeta	1	1
Turbellaria	7	5
Pisces	2	2
Insecta		
Coleoptera	4	4
Diptera		
Ceratopogonidae	2	2
Chironomidae	12	14
Culicidae	43	50
Simuliidae	14	9
Tabanidae	1	1
Ephemeroptera	3	3
Odonata	4	4
Trichoptera	2	3

Laboratory infection studies have also been conducted to determine host specificity (Table 2). In these tests, a few *Nosema* species showed little host specificity, but most species of *Nosema*, and particularly *Stempellia* and *Thelohania*, were found to be host specific. Many *Nosema* species of lepidopterans readily invaded a variety of host species of this order, however, only four species (*N. algerae*, a *Nosema* species from a mite and two *Nosema* species from two species of *Ishnura* [damsel

flies]) caused pathologies in animals in orders other than those of their natural host. The *Nosema* from mites readily produced light infections in *A. quadrimaculatus* larvae; however, the other three *Nosema* species attacked only the larvae of *H. zea* (corn earworms) and then only after the worms had been starved for 24 hr or longer.

TABLE 2. Experimental Transmission Tests with Microsporida in Ten Insect Species

Microsporida	Hosts: *Aedes aegypti* (L.)	*Anopheles quadrimaculatus* (Say)	*Culex pipiens quinquefasciatus* (Say)	*Goeldichironomus* sp.	*Musca domestica* (L.)	*Heliothis zea* (Boddie)	*Trichoplusia ni* (Hübner)	*Paramyelosis transitella* (Walker)	*Blattella germanica* (L.)	*Solenopsis geminata* (Fabricius)
Nosema algerae (Vávra and Undeen)	+	+	+	+	+					
N. apis (Zanders)										
N. heterosporum (Kellen and Lindegren)						+				
N. invadens (Kellen and Lindegren)								+		
N. kingi (Kramer)										
N. necatrix (Kramer)						+	+			
N. plodiae (Kellen and Lindegren)								+		
Nosema sp. from *Arzama densa* (Walker)						+	+			
Nosema sp. from *Carpocapsa pomonella* (L.)										
Nosema sp. from *Crangonyx* sp.										
Nosema sp. from *Ishnura* sp.						+				
Nosema sp. from *Ishnura* sp.						+				
Nosema sp. from *Neophelodes emmedonia* (Cramer)						+			-	
Nosema sp. from a mite (Kudo)		+				+	+			
Parathelohania anophelis from *A. quadrimaculatus* (Say)										
P. obesa from *Anopheles crucians* (Wiedemann)						-				
Stempellia sp. from *C. p. quinquefasciatus* (Say)			+							
Stempellia sp. from *Chironomus attenuatus* (Walker)						-				
Stempellia sp. from *G. holoprasinus* (Goeldi)	-			-		-				
Stempellia sp. from *Uranotaenia sapphirina* (Osteen-Sacken)	+									
Thelohania opacita from *Culex salinarius* (Coquillett)							-			
T. tabani Gingrich from *T. atratus* Fabricius							-			

Subsequently, three of the *Nosema* species that showed exceptional virulence in their natural host were chosen for infection studies with crayfishes, fresh-water shrimps, and mosquito fishes. The microsporidans used were *N. necatrix*, *N. algerae*, and an undescribed *Nosema* from *A. densa*. Corn earworms heavily infected with each of these microsporidans were fed to the test animals periodically. None of the test animals showed symptoms of infection during a 60-day test period, thus mortalities in these animals were no greater than in animals used in control tests. Spores of *N. algerae* were also fed to chickens, damsel fly naiads, dragon fly naiads, dytiscid larvae, helgramites, and mice. None of the animals developed gross disease symptoms, and no evidence of nosematosis was found in histological preparations of dissected tissues from these animals.

Our field observations and laboratory infection studies, therefore, have demonstrated some measure of natural host specificity of microsporidans in both aquatic and terrestrial animals; those in aquatic animals appear to be more host specific, especially species of the genera *Stempellia* and *Thelohania*. Many microsporidan species infected only species of one family or order; few species crossed host classes. Nevertheless, starved corn earworm larvae are susceptible to *N. algerae* while nonstarved larvae are more difficult to infect. Thus, when starved for 24 hr or more, they provide a good system for mass culture of many *Nosema* species from insects.

Since fishes of the genus *Gambusia*, crayfish, and fresh-water shrimp were not susceptible to *N. algerae*, *N. necatrix*, or the *Nosema* species from *A. densa*, even though all three are virulent pathogens in their natural hosts, these three species of *Nosema* may not be hazardous to other nontarget animals. This apparent host specificity of certain species of Microsporida from aquatic invertebrates will be documented further in manuscripts that describe new genera restricted to the species of certain families of aquatic animals.

SUMMARY

Some of the Microsporida are promising biological control agents for aquatic pest species, and most species do not appear to cause pathologies in nontarget animals other than insects. Each new microsporidan found in aquatic pests, however, should be tested on nontarget animals since degree of host specificity is variable among species. Since new information indicates that some microsporidans cause congenitally transferred diseases in mosquitoes, they may be useful for control of mosquitoes. Basic studies pertaining to development of these microsporidans in female hosts carrying the disease via the ovaries and eggs are needed. The search for new and more efficient microsporidans in pest and disease vectors of tropical regions will be continued since many of these appear to invade their host per os.

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DISCUSSION

J. E. HENRY: We have been working for a number of years with a microsporidan, *Nosema locusti*, in grasshoppers and we have about 8 years of practical field experimentation behind us. In many areas, we've gone a little further than Dr. Hazard has with his, and perhaps this is the reason I'm here. I was surprised that our dosage rates for field application are quite similar to those of Dr. Hazard. Our standard dosage is a billion spores in a pound and a half of wheat bran per acre, and this is quite close to what the people at the Insects Affecting Man and Animals Laboratory were using in the Canal Zone against *Anopheles* mosquitoes. We find that these dosages invariably will cause 50% mortality in the grasshoppers within 4 weeks after application, prior to oviposition, and then 30-50% of the residual grasshoppers will be infected, inhibiting or reducing reproduction. *Nosema locusti* infects well over 60 species of grasshopper, and it will also infect the Mormon and black field crickets. Attempts to infect the eastern house cricket, however, have failed. Also, it will not infect honeybees. We've done extensive safety tests with *Nosema locusti* and this may apply perhaps indirectly to what Dr. Hazard is doing. We've done acute dermo-toxicity, acute oral-toxicity, inhalation studies, long-duration feeding studies, and skin irritation studies in rats, but in all cases none became infected. These studies were in cooperation with Dr. Arthur Heimpel at Beltsville, Maryland, under contract to a private laboratory. In no instance did we find any pathologies in rats. We also bioassayed all rat tissues or samples of rat tissue and their gut contents, in grasshoppers after the test. We found spores in rat gastric contents, in the rats that were killed immediately after treatment with the acute oral toxicity treatment. These treatments were 10 times our normal dosage per acre. When first tested, we also found one rat with a small concentration of spores that was sacrificed three days after treatment--these spores were pretty well disrupted and the animals were not infected. But for the ones we got at 0-day time that were infected and sacrificed immediately after treatment, we checked the spores, and on the first run-through they were not infective. We went back to find out why, and found some infectivity in those spores after cleaning them up from the gastric contents of the rats.

We've also tested in fish, and found no pathology there. So that's about all I want to say; if you want to know more about the grasshopper work, I'll talk to you later. As it relates to safety, I feel that *Nosema locusti* at least is safe, but as mentioned before, each organism will be tested on its own merits.

IGNOFFO: That was the major point in bringing Dr. Henry here, since to date that is the only available systematic test of a known organism against mammalian systems.

JOE MADDOX: Most of the work that we do with Microsporida has also been in terrestrial arthropods. But I would like to make a few comparisons. We are working with a few aquatic insects infected with Microsporida and I'd like to make some comparisons between what we've found in aquatic and terrestrial microsporidans. Dr. Hazard mentioned that he had about 120 microsporidan species and many of these, I suppose, are undescribed. But we've found many microsporida stored away because the order is in such a state of confusion, as far as taxonomy and classification are concerned. I would imagine if this is clarified in the near future there will be a vast increase in the number of described species.

We found, for example, that some of the key taxonomic characters (and I've talked to a number of people about this over the years) vary as a result of environmental conditions. For instance, in one species I can vary the temperature at which the host is maintained; at low temperatures it falls into one genus, at high temperatures into another. We have another species and this same thing happens, except that it's a function of tissue and time. If the insect goes into diapause, certain tissues develop forms which are in completely different genera and yet this, I feel certain, is one species. I won't elaborate on how we determine that. We also find that some microsporida, when put into unusual hosts, will infect tissues that they don't infect in their original host--they go into these tissues and act differently. So we have some very confusing aspects of classification within the microsporida that just have to be clarified before many of these species can be described. Fortunately, most of the microsporida we have examined have stable base characters. One noticeable difference between microsporidan-infecting terrestrial insects and aquatic insects is the ability to be stored in liquid nitrogen. We store all of our terrestrial microsporida in liquid nitrogen and have done so indefinitely with no significant loss of viability. Some have been stored now for over six years and in the ones that we can bioassay we can't detect any differences in those that have been stored versus the fresh microsporida. However, Al Undeen has done this with *Nosema algerae* and we've done this with some other microsporida from chironomids. They won't withstand storage in liquid nitrogen. I don't know whether this just happens with the ones we're looking at, or whether this is a unique difference between microsporida-infecting aquatic animals versus microsporida-infecting terrestrial ones. We found the same to be true with drying. Most microsporida from terrestrial insects can withstand drying, some for

considerable periods of time. Those from aquatic insects that we worked with could not withstand drying, even for very short periods. This is not surprising, since freezing is a sort of drying process.

I think we need to define very carefully what we really mean when we say host range. For example, we can frequently infect insects with a microsporidan if we feed it to them in very large doses as newly hatched instar larvae. If you feed it to them as second or third instar or older larvae, infection can't be achieved unless you define very carefully what stage you're trying to infect and how to go about infecting it. These host range statements don't mean a lot as far as host ranges go, but in relation to what's happening in the field, they're significant. In other words, if you can't infect a later instar larva with microsporida, the chances are that particular species are not going to be infected under field situations or conditions. Frequently we can infect insects by injecting into the hemocoel infectious stages, infectious spores of which the polar filaments have been extruded or vegetative forms from another insect. But for some of these microsporida you can expand the host range enormously--it'll go into almost any arthropod you inject it into if you inject the correct stage. Others have a very specific host range and even by injecting you can't get it into other arthropods. Thus, I think we need to look at some of the aspects of host range and what really determines host specificity.

AL UNDEEN: In the Biology Department of the University of Illinois, we've been studying *Nosema algerae*, which is probably the same microsporida that Dr. Hazard has discussed. We don't know for sure, we've never really compared them that closely. We work with *Anopheles stephensi* since this microsporida infects better in *A. stephensi* than it does in *A. albimanus*. The required infective spore dose per unit area is about the same as Dr. Hazard described in his *Nosema algerae* field tests. We have been able to infect everything we've injected with this microsporida, we've injected the spores in the hemocoel, anything that would become infected, and this has produced a good method for mass producing spores. The feeding tests we've done in the past have been with larger larvae, those that are easily handled, nymphs of aquatic insects and that sort of thing; we've not been able to get infections by feeding spores to these insects. However, more recently we have fed Heliothazia larvae as first instars, without starvation or anything else, and they were all infected. So I think, as Dr. Maddox has stated, that we really need to look at the host range of this a little more closely.

I want to talk about the safety of these microsporida and vertebrate systems. We've used pig, bovine, and horse kidney cells in tissue culture and they served as adequate hosts for *Nosema algerae*. This is simply inoculating the spores directly into the tissue culture medium, with the cells as a monolayer on the bottom of the flask. This *Nosema* developed at 26 and 35 C with production of viable spores, and at 37 C, *Nosema algerae* appeared to multiply for a short time and then die. No infected cells were seen at 38 C. We are presently examining the

possibility of a vertebrate nosematosis resulting from spores injected by mosquito while obtaining a blood meal. I haven't examined the probability of this event happening, however, we have seen infected salivary glands in female *Anopheles stephensi*, and *Nosema algerae* germinate very well in mouse and human blood plasma.

Since 37 C appeared to be the upper limit of this *Nosema*'s temperature tolerance, mice were injected in areas suspected to be cooler than the interior body temperature. About one million spores were injected at each site under the skin of the tail, the ears and the hind feet of white laboratory mice. At 2, 3, 4, 6, 8 and 12 day intervals a mouse was killed, the skin over the injection site excised, and the subdermal layer scraped to obtain a microscope slide which was stained with giemsa blood stain. *Nosema* developmental stages were found at each injected site, through the 8th day after injection. No parasites were found on the 12th day. And no *Nosema* was found at any uninjected site, so this means that the *Nosema* did not spread throughout the mouse and it also means that there wasn't any other *Nosema* present in the mouse before it was injected. So, the host range in *Nosema algerae* seems entirely dependent upon its ability to invade the host. Since insects do not have the same degree of resistance to invasion throughout development, susceptibility tests must include the whole period of exposure to *Nosema* spores. The immunity system of the vertebrate is an additional factor in attempting to destroy the *Nosema* once invasion has occurred, that is, in the vertebrates. Experiments using cortisone to suppress the immunity system are planned in order to see how severe a *Nosema* infection can become in an immunologically incompetent individual. I don't know whether these results can be generalized to other microsporida but I think it is here that these guidelines for safety standards are really needed.

IGNOFFO: You might want to use very young mice, too, in addition to the immuno-depressant drugs.

MARSHALL LAIRD: I'd like to try a red herring at this point, arising from the remark about giemsa bloodstain and the staining of microsporidans. Those of us who work with mosquitoes will be well aware that we have customarily fed mosquito larvae on dog biscuit. Now, if you think about that, it does seem a little strange and it did lead to tiny amounts of DDT sprayed onto alfalfa, getting into dog biscuit, and then into mosquitoes, which allegedly have never been exposed to DDT, some years ago. I mention this because eosin stain is the easiest of all stains to use. At the same time, it gives infinitely variable results and these are attested to by the fact that there is a literature of several hundred papers on yet another method of attaining success in staining with giemsa. It seems to me that if we really are going to get into the nitty-gritty of, for example, the Microsporida, it might perhaps be worthwhile casting around and endeavoring to develop some staining methods that are actually tailored to the microsporidans instead of picking a stain from another group.

UNDEEN: The giemsa stain as I use it did show developmental stage of the microsporida in which the cytoplasm and nuclei could be clearly seen. This was different from any other tissue I saw in the mouse slides or in the mosquito slides; it was also identical to the stages seen when taken from mosquito tissue or any other insect into which it was injected.

IGNOFFO: What was the source of nutrients for the development of the spores I assume you used? And how far was the stage developed? Did it just germinate?

UNDEEN: In the mouse it was difficult to tell just how far it went because I gave them an inoculant, the temperature was high, the development was rapid, and I suspect the spores I saw on the slides could have either been produced in the cells of the mouse or could have been from the original inoculant. I had no way of determining this. However, I did see the developmental stages which could only have come through growth from the spores.

IGNOFFO: The basic question I was getting at is, was the habitat just a nice localized place for development of the spores or, in fact, did spores develop on mammalian host tissue?

UNDEEN: I've never seen the microsporida in the tissue culture or in the insects to develop outside of the cell, so I imagine the development was intracellular as it was anywhere else and it must have been within a mouse cell. The spores I injected were treated to remove any extraneous amount of insect tissue. The spores were cleaned with a density gradient and several changes of distilled water, so I'm sure they must have been within the mouse cells. You had to scrape the tissue and treat it pretty roughly in order to see anything so I assume you had to break open the cells to find anything. Again, this is preliminary work and I'm not sure if all aspects of the development occurred.

IGNOFFO: I think in most systems if you work hard enough and put no restriction on dose or what site of infection to use, you probably can obtain considerable material. Now how, then, does that relate to specificity? This is the question we'll have to address ourselves to. You can go to the literature on viruses and bacteria and you find many instances of cross-transmission, using roots of administration that are far from the natural. I think in most of these situations what you're trying to do is establish if, in fact, the organisms can survive in those systems. Another question is, can you retrieve the organism in an infective stage and place it back into the normal host?

HAZARD: We have looked for a natural transmission field because of some of the difficulties we have, mainly insufficient personnel for all

these laboratory studies. Also, while we're collecting these non-target organisms we have a chance to pick up other microsporidans which we can add to our list and help us in our taxonomy, and this is one of our more important studies right now. We've got to find out what we're working with. We don't see this in the field, and we couldn't find any infections. If there are any infections it's going to be less than one percent. We brought back numerous specimens to section and examine more closely, but I wonder in all these animals that you injected, did you run this material through ludox? This is going to pretreat these spores if they're going to extrude, no matter what you put them into.

UNDEEN: The ludox pretreatment, for those of you who don't know what it is, is a silica colloid, used in floor waxes and stuff. I use it as a density gradient for separating microsporida spores from trash and mosquitoes, or whatever insect I separate it from, but I use it mainly in this instance for getting the microsporidan free of the bacteria, because if you run it through this gradient, all the bacteria are at the top and the microsporida is in the band down by itself. Under some conditions in vitro it does accelerate germination of the spores, however, the untreated spores that were not run through the ludox gradient do extrude, or germinate, in blood and in the tissue culture medium. The spores germinate, whether they're run through the ludox treatment, in blood, serum, from these animals.

IGNOFFO: I think what they're trying to stress is that there are techniques in which you can handle the pathogen, handle the host and manipulate it in such a way that you can get data. I think that's a good approach and should be considered. It's been noted that there seems to be a limit in terms of upper temperatures at which these organisms will complete their development to the spore stage. But we also have to consider the opposite area, that is, there are many vertebrates existing in nature in which the body temperature is well below 37 C. So the potential for infection will have to be looked at on the basis of the host and the pathogen. The requirements are going to be slightly different. We can establish guidelines which will assist us, but the criteria for measuring the effects will be different depending upon the host and pathogen.

PERSISTENCE OF PATHOGENS IN THE AQUATIC ENVIRONMENT

Y. Tanada*

INTRODUCTION

The persistence of pathogens in the aquatic environment is an area of insect pathology that has received very little exploration. This is especially true when pathogens, which attack terrestrial insect pests, occur in an aquatic environment. [Terrestrial insects are defined as insects which spend or complete all of their life cycles independent of a body of water. Aquatic insects are those which spend the entire or a portion of their life cycles in or on a body of water.] A list of factors that may affect the persistence of pathogens in the aquatic environment is given in Table 1.

TABLE 1. Factors Affecting Pathogen Persistence in Aquatic Environment

Physical	Chemical	Biotic
Current (waves, tides, etc.)	Solutes:	Fauna
Density	Acids, alkalis (pH)	Flora
Depth	Gases (O_2, CO_2, etc.)	
Desiccation	Inorganic salts	
Sunlight (UV)	Organic compounds	
Pressure	Suspended Insolubles:	
Salinity (osmotic pressure)	Inorganic compounds	
Substrate (bottom, shoreline)	Organic compounds	
Temperature		

A major difference between a terrestrial and an aquatic environment is the medium, air or water. In the former, the pathogens are less likely to remain suspended in air unless moved by air currents and other forces. However, in an aquatic environment, the size, buoyancy and swimming appendages of the various pathogens may enable them to remain suspended and move about in the medium for long periods. The role

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played by noxious compounds and contaminants in the terrestrial environment varies from rapid dispersion in the air to prolonged persistence in the soil. In an aquatic habitat, the contaminants in water may remain for long periods depending on the rate of breakdown, dilution, and removal by water movements. Thus, pathogens in the aquatic environment may be in contact with these contaminants for long periods.

Pathogens of terrestrial insects experience two physical factors that play major roles in their persistence. These are sunlight, especially the UV spectrum, and temperature. These factors play less significant roles in the aquatic environment, where UV would have a minimal effect, and temperature, except in hot springs and volcanic pools, would be usually below air temperatures and at the minimal, not many degrees below freezing. On the other hand, the aquatic environment has factors, such as pH, osmotic pressure, dissolved inorganic and organic solutes, and water movement that may seriously affect the pathogen persistence.

In the terrestrial habitat, the microenvironment that closely approximates the aquatic environment is that of the soil, especially after prolonged wetting due to rainfall, irrigation, etc. The microenvironment of soil at its maximum moisture-holding capacity would be similar to that of the aquatic environment, especially the sediment along the banks of streams, rivers and lakes.

PERSISTENCE OF PATHOGENS OF TERRESTRIAL INSECTS

Inasmuch as there is a serious lack of information on the persistence of pathogens of terrestrial insects in the aquatic environment, it is necessary to extrapolate from laboratory studies and speculate on the survival of such pathogens in an aquatic environment. In some cases, a pathogen of a terrestrial insect has been transmitted to an aquatic insect, and such a pathogen, therefore, may become involved in an aquatic environment.

VIRUS

Viruses of terrestrial insects maintain their infectivity for one or more years when stored in water under refrigeration (4 C). Such viruses are mainly the occluded viruses, and they are stored at the stage of occlusion within the inclusion or occlusion bodies. The nuclear polyhedrosis virus (NPV) of *Bombyx mori* has been maintained for 20 years in hemolymph in flame-sealed glass tubes held at refrigeration temperatures (45). Cunningham, however, has observed, using the electron microscope, that prolonged storage for 6 years at 4 C causes holes to appear on the polyhedra of the NPV of *Lambdina fiscellaria fiscellaria* and the pathogenicity of the virus is greatly reduced. A similar observation has been made with the scanning electron microscope on the polyhedra of the NPVs of *Galleria mellonella*, *Hyphantria cunea*, and *Bombyx mori* (60). After several months to years of storage, the surface of the polyhedra

deteriorates, shows cavities and exposes the virions.

The insect pox viruses within their spherules, as in the case of the NPVs, may persist under refrigeration. The survival of the granulosis viruses within the capsules is, in general, for not more than 1-2 years when suspended in water and held in the refrigerator. We have maintained the activity of the *Tipula* iridescent virus for about a year under refrigeration. There is very little information on the persistence of other nonoccluded viruses in aqueous suspension.

Earlier it is noted that the soil of the terrestrial environment may closely approximate that of the aquatic environment. This aspect is discussed, especially with insect viruses, because extensive studies have been conducted along these lines by various workers (50, 51). The insect granulosis virus (GV) of *Pieris brassicae* cannot be readily washed out of soil and sand (9). The prolonged persistence of viruses in the soil throughout the year even after rainfall has been reported (21, 23, 25, 52). The NPV of *Trichoplusia ni* apparently persists for more than 4 years in the soil (24), but the GV of *T. ni*, after 2 years, occurs only in small amounts in the soil. The NPVs of *Hyphantria cunea* (20) and of *T. ni* (54) accumulate less than 5 cm from the soil surface and polyhedra have been demonstrated in spaces between the soil particles (20).

The soil pH may play a significant role in persistence of the NPV of *T. ni* (55). In a loamy sand of various pH (4.83-7.17), the lower the pH the more rapidly the virus is inactivated. This would be expected, since the virus polyhedra are soluble in weak alkali and acids.

BACTERIA

Both nonspore-forming and spore-forming bacteria would be expected to live for short periods in an aqueous environment, but this would vary with the availability of nutrients, pH, toxic substances, temperature, etc. With spore-formers, the presence of water would increase the likelihood of germination and thereby may reduce the persistence of the bacteria. The spores of *Bacillus popilliae*, when suspended in water and kept for 20, 40, and 80 days, progressively lose their potency with time (4).

Some pathogenic crystalliferous bacteria, e.g., *Bacillus thuringiensis* and its varieties, have a crystalline parasporal body, the delta endotoxin, that is highly toxic to certain insects. The endotoxin is proteinaceous and is soluble in weak alkali. In an aqueous environment with high pH, the endotoxin may be destroyed.

The most favorable physical environment for bacteria of terrestrial insects is the soil. Both nonspore-formers and spore-formers have been reported to persist in the soil. Some facultative bacterial pathogens may propagate in the soil. The common bacterium, *Bacillus cereus*, has strains which are pathogenic to insects. With milky disease bacteria, these obligate bacteria infect soil insects in nature, and have been

applied to the soil and have persisted and controlled scarabaeid beetles for many years. Most studies have been conducted with *Bacillus popilliae*, the Type A organism of the Japanese beetle, *Popillia japonica* (4, 69). The spores of *B. popilliae* tend to remain in the upper layers of the soil, the first one inch (4), but there is some vertical spread to a depth of 4 inches. After 18 weeks, there is some loss in the capacity of the soil to produce disease, which suggests a decrease in the persistence of the spores. However, the loss of effectiveness is due more to a diluting or leaching factor than to an actual mortality of bacteria. In field-collected soil, Beard (4) reports that 90% of the grubs become infected in soil in the top inch, 54% in the second inch and 46% in the third inch. Low soil pH (>6.0) may adversely affect the spores of *B. popilliae*, and high pH (<6.0) may increase the potency of the bacterium.

Several Rickettsiae have been reported from larvae of scarabaeid beetles which live in the soil. These Rickettsiae produce infections in larvae and may be presumed to persist in the soil.

FUNGI

Most fungi of terrestrial insects, as with bacteria, may not persist for long periods in an aqueous environment. The presence of moisture increases the likelihood of germination of resistant and non-resistant spores, and only those capable of saprophytic growth would be expected to survive in the absence of hosts.

Some entomogenous fungi are known to persist for long periods in the soil, not only in resistant stages, but the facultative form also may grow saprophytically in the soil. This may occur with the green muscardine fungus (*Metarrhizium anisopliae*) and the white muscardine fungus (*Beauveria bassiana*), both of which are known to infect soil insects under natural conditions. Species of *Cordyceps* are known to infect soil insects, but there is very little information of the mode of infection and persistence in the soil.

PROTOZOA

The Protozoa are generally maintained in the laboratory in water and under refrigeration. This is especially true with spores of Sporozoa, which may remain viable in water at 2-4 C for more than a year (31, 63, 64). The clean spores of the microsporidan, *Nosema bombycis*, remain viable for more than 8 years when stored in a refrigerator (64). At 15 and 25 C, microsporidan spores are viable for a month or two in water or saline. The spores of *Octosporea muscaedomesticae* in water at 5 C remain viable for at least 2 years (31). The spores of *Nosema necatrix* persist for only 6 weeks at -34 C, and at 4 C are viable for 2-1/2 years in water suspension, but there is a marked reduction in infectivity (Table 2) (33).

TABLE 2. Viability of *Nosema necatrix* Spores Stored in Water at 5 C*

Length of Storage	Reduction of Infectivity
1 day	0
6 months	100 X
1 year	1,000 X
2 years	10,000 X

*Maddox, 1973.

The polar filament of a microsporidan spore is used to inoculate the planont or sporozoite into the host cell or hemocoel and its extrusion is affected by certain chemicals, pressure, and pH. High K content and a pH 10.8 favor release of the polar filament (22, 36). The effective range of pH also varies with different cations (22), which may cause premature release and death of the planont when no host is available. The pressure developed at great depths of lakes and oceans may also force the extrusion of the polar filament.

When kept moist throughout storage, the microsporidan spores can persist in water, but if they are first dried and then wetted, this may cause the ejection of the polar filament. This occurs with the dried spores of *Nosema whitei*, which when placed in water readily extrude their polar filaments (30, 31).

The flagellate, *Leptomonas ctenocephali*, of the dog flea, *Ctenocephalus canis*, becomes nonmotile in salt concentrations of 4% and over, but will revive if salinity is reduced to below 4% (13).

Several protozoans, especially the sporozoans, infect soil insects, and they would be expected to persist in the soil. The larvae of several species of lepidopterous insects of alfalfa develop a microsporidan infection when fed the soil from the alfalfa field (52).

NEMATODES

Temperature and moisture appear to be the major factors affecting the persistence of nematodes (40, 62, 67). Nematodes are capable of surviving in water but their length of survival may depend on temperature, salinity, oxygen, pH, and other factors. The nematode, DD-136 (*Neoaplectana carpocapsae*) can be maintained for up to 5 years in water receiving periodic oxygenation and held at 7.1 C (11). It is destroyed at temperatures above 38-45 C (10, 66) and at freezing temperatures in water (66). It can survive the pressures of a conventional power sprayer (200-300 psi) and can be safely combined with common insecticides and fungicides (10).

A survey of the natural distribution of *Neoaplectana* indicates that insect parasitism is greatest at high soil temperatures and with

abundant soil moisture (40). There may be a preference for calcareous soil by mermithid nematode pathogens of insects (53). The mermithid nematode of the ant, *Pheidole pallidula*, appears to be associated with a particular soil structure (61). The author has been able to predict where the nematode may be found by the geological structure of the soil.

Certain fungi (Saprolegniales and Chytridiales) and protozoans (Actinomyxida) are known to parasitize free-living stages of mermithid nematodes, and they may also attack the entomophilic nematodes (40).

PERSISTENCE OF PATHOGENS OF AQUATIC ENVIRONMENT

The persistence of pathogens of aquatic insects will be treated under two major categories: (1) persistence in the fresh water habitat and (2) persistence in a marine, including estuarine, habitat. Inasmuch as there are only a relatively few insects in the marine habitat, the pathogens of some invertebrates other than insects will be discussed in this section. Under both habitats, the discussion will be centered on the physical, chemical and biotic factors that may affect the persistence of pathogens. These factors are listed in Table 1, but some of them will not be considered because of a lack of information.

Most pathogens of aquatic insects are microsporidans, fungi and nematodes, with a lesser number of bacteria and viruses. Thus the discussion below will be directed largely toward the first three pathogens.

PHYSICAL FACTORS IN FRESH WATER HABITAT

Since influence of the current or water movement is closely associated with the density (buoyancy) of the pathogens, these two factors will be considered together. A buoyant or suspended pathogen would be expected to be carried or moved about by water movement. Pathogens less than one micron may remain suspended in water. Larger pathogens may have structures or appendages (cilia and flagella) that enable them to move about in water. Still others, e.g., microsporidans, have spores with caudal appendages, needle-like structures, and gelatinous capsules that enable them to float (64, 65).

The effect of depth on persistence of pathogens has received very little study. The rate of infection by the mermithid nematodes, *Hydromermis contorta* and *Limnomermis bathybia*, on the midge, *Chironomus modestus*, is inversely correlated with depth (34), being highest at 0.5 m and lowest at 3-5 m.

In general, desiccation would have drastic effects on the persistence of most pathogens in the aquatic environment. However, in some instances the pathogens are able to resist desiccation and infect the insect host when water becomes available again. Chapman and Glenn (5) in their study on the incidence of the fungus, *Coelomomyces punctatus*, have reported its reappearance in larval populations of *Anopheles*

crucians after an absence of 29 weeks, caused principally by a lack of water in the ponds. In other situations, alternate wetting and drying may favor the pathogen. The spores of the microsporidan, *Thelohania californica*, a pathogen of *Culex tarsalis*, when freshly removed from infected mosquitoes are not infectious, but become so when alternately dried and hydrated (28).

The sporangia of *Coelomomyces* spp. can be stored at 5-10 C on moist filter paper for 5-8 months (7, 68). The germination of these sporangia is markedly affected by temperature (7), with no germination below 10 and above 35 C, and optimum germination at 23 C. Germination occurs in tap and distilled waters, rain and lake waters, in pure clean water and in water contaminated with bacteria and protozoa (7). Earlier workers experienced difficulty in studying this group of fungi because of failure of sporangial germination. Roberts et al. (41) state: "The inability to induce dehiscence upon demand has been a major deterrent to the study of most *Coelomomyces* species." They report that the dehiscence of the sporangia of *C. psorophora* from *Aedes taeniorhynchus* decreases from 23% dehiscence to 3% in less than 1 and 1.5 months when stored in water at 10 C. They also note reduced dehiscence with use of moist filter paper at 10 C as reported earlier (7, 68). Roberts et al. (41) tested the dehiscence of *C. psorophora* with various salts, reducing agents, chelating agents, buffers, alcohols, carbohydrates, fatty acids and derivatives, amino acids and derivatives, peptides, amines, purines, pyrimidines, antibiotics, and plant hormones. The most active substance is Tris [tris (hydroxymethyl) amino-methane] at pH 8.9 and at 1-20 mM. The active compounds have a basic requirement for $-NH_2$ and either $-COOH$ or $-CH_2OH$ attached to the alpha carbon, but only certain amines and amino acids are highly active. Sporangial preparations free of host debris are not responsive to Tris, but addition of bacteria-free homogenates of *A. taeniorhynchus* larvae reactivates the material.

Salinity would be expected to be one of the major factors affecting persistence of pathogens of fresh-water insects. Data in this area appear to be seriously lacking. The habitat of a mermithid nematode, *Romanomermis* sp., on the southern house mosquito, *Culex pipiens quinquefasciatus*, may be limited by salinity (37). The mermithid does not parasitize the mosquito when held in water with a NaCl concentration above 0.04 M (Table 3). A sharp drop in parasitization is evident at concentrations between 0.015 and 0.030 M NaCl. This suggests that the mermithid will not survive under a high salinity.

BIOTIC FACTORS IN FRESH WATER HABITAT

Many pathogens of terrestrial insects commonly persist in the biotic habitat (48, 49, 50). Such persistence occurs in the primary and secondary hosts, parasites, predators, and other animal carriers. There is increasing evidence that this also is true with pathogens of aquatic insects. The host-parasite relationships of several microsporidans (*Thelohania* spp.) have been studied in mosquitoes and four basic types of relationships based on tissue specificity, sporogonic cycle to

the sex, and on the expression of patent infections of the host have been classified (29). The latter workers point out that in the Type I relationship, the transovarial transmission of the microsporidan through the female parent, which is not killed, enables the microsporidan to persist throughout the year in the hibernating eggs of the univoltine mosquito. Since the host larva, pupa and adult are absent during the 11-month period, it is suggested (29) that the microsporidan spores will not persist in the environment in the absence of hibernating eggs. In hibernating hosts, development of a microsporidan ceases or is greatly delayed (63).

TABLE 3. Effect of NaCl on Parasitism of *Culex pipiens quinquefasciatus* Exposed to Constant Numbers of *Romanomermis* sp.*

NaCl (M conc.)	Percent Survivors Infected		
	Trial 1	Trial 2	Trial 3
0	60	90	76
0.005	--	87	75
0.010	33	74	64
0.015	--	49	51
0.020	6	32	28
0.030	0	11	9
0.035	--	2	2
0.040	2	0	1
0.050	0	0	0
0.060	0	--	--
0.080	0	--	--

*Peterson and Willis, 1970.

Transovarial transmission and subsequent persistence in the host also occur in the mosquito iridescent virus infecting *Aedes taeniorhynchus* (16).

There are increasing numbers of instances of pathogens infecting several species of aquatic insects. The iridescent virus discovered in the rice stem borer, *Chilo suppressalis*, can be transmitted per os in the laboratory to the following aquatic genera: *Aedes*, *Anopheles*, *Culex*, *Culiseta*, and *Psorophora* (12). On the other hand, the mosquito iridescent virus is infectious only for *Aedes* and *Psorophora*. The fungus, *Coelomomyces macleayae*, infects tree hole mosquito larvae of 3 *Aedes* subgenera in Australia, Fiji, and the United States (35), and also attacks the predator mosquito, *Toxorhynchites rutilus septentrionalis*. A virulent aquatic fungus, *Lagenidium* sp., was isolated from *Culex restuans*; it also infects several other species of mosquitoes, *Anopheles quadrimaculatus*, *Anopheles* sp., and *Psorophora* sp. (58).

Several fungi which predominantly infect terrestrial insects also infect aquatic insects. *Beauveria tenella* (*B. bassiana*) infects *Aedes sierrensis*, *A. aegypti*, *A. dorsalis*, *A. hexodontus*, *A. pipiens*, *Culex tarsalis*, and *Culiseta incidens* (39). A possible strain of the ubiquitous green muscardine fungus, *Metarrhizium anisopliae*, is highly virulent for the mosquito larvae of 3 genera, *Anopheles*, *Culex*, and *Aedes* (47). The fungus, *Fusarium oxysporum*, infects *Aedes detritus* and *Culex pipiens pipiens* (17).

The microsporidan, *Nosema algerae*, isolated from *Anopheles stephensi*, infects other species of *Anopheles* (59). It is also infectious for crayfish, whose gills are most heavily infected. The mermithid nematode, *Hydromermis contorta*, has several physiologically suitable hosts, *Procladius denticulatus*, and four species of *Tanitarsus* (34).

Some pathogens serve as food for other animals in the aquatic environment. The infective larvae of the nematode, *Neoaplectana carpocapsae*, are fed upon by larvae of *Simulium vittatum* and *Culiseta inornata* (62). The former feeds upon the nematode faster at 10 C than at 20 C, and the latter eats more at the higher temperature. The sporangium of the fungus, *Coelomomyces punctatus*, is eaten by ostracods, rotifers and other small invertebrates, and is destroyed by parasitic fungi (6). The invasion of the ciliate, *Tetrahymena pyriformis*, into the larva of *Culex tarsalis* is affected by the presence of the larvae of *Aedes aegypti*, which may be consuming the ciliate (14).

PHYSICAL FACTORS IN MARINE HABITAT

During the past several decades, there has been a great increase in the number of studies on diseases of marine animals, but these studies are widely scattered in the literature (42). Most of the reports are concerned with the taxonomy and biology of the pathogens, with only limited studies on the epizootiology of the diseases. I have not made an exhaustive search of the literature on the persistence of pathogens of marine invertebrates and my review is far from complete.

In the terrestrial environment, sunlight, especially the UV radiation, plays a major role in reducing the persistence of pathogens in the host habitat. However, in the aquatic habitat, penetration of the UV radiations into the water is limited, and therefore is relatively ineffective. This is indicated in the rearing of bivalve mollusks where the UV treatment of water and phytoplankton greatly reduces the incidence of infection of mollusks by fungi (32). Certain soluble compounds, such as antibiotics, phenols, insecticides, weedicides, and such inert substances as silt and kaolin may improve the growth rate of bivalve larvae. Loosanoff and Davis speculate that these compounds remove the toxins from water or assist in killing pathogenic and other competing microorganisms (32).

Salinity may be a significant factor in the persistence of pathogens in the marine and estuarine habitat. Johnson and Sparrow (27) state: ". . . The status of knowledge on fungi in salt water is such

that while considerable is known about their structure and occurrence, the mechanisms permitting them to exist in a 'salty environment' are not understood or at most only dimly apprehended." This statement would also apply to other pathogens of marine invertebrates. The fungus, *Lagenidium callinectes*, which infects the blue crab, *Callinectes sapidus*, has a wide tolerance range of salinity and can sporulate in water of 5-30 ‰ salinity (26). The mosquito, *Aedes australis*, is capable of breeding in pools ranging from fresh water to hypersaline, but the infection by the fungus, *Coelomomyces opifexi*, is associated with less than approximately 20 ‰ salinity and more particularly below 10 ‰ salinity (38). The high concentrations of sea salts inhibit the dehiscence of the fungus sporangia, whose walls become thicker and darker as a result of exposure to higher salinities. The germination and zoospore activity of the sporangia have been studied at various temperatures and different salt concentrations (Table 4) (38). Germination occurs at 5, 10, 23 and 28 C in distilled water, brackish water (4.2 ‰), and in salinities of 8.5 ‰ and lower. Zoospore activity decreases at 5 and 10 C. Regardless of temperature, germination is variable at the higher salinities, but none occurs in pure sea water (35 ‰).

TABLE 4. Mean Averages of Sporangial Germination of *Coelomomyces opifexi**

Medium	Temperature			
	5 C	10 C	23 C	28 C
Distilled H_2O	1/5**	1/5	4/5	4/5
Tap H_2O	4/5	2/5	4/5	4/5
Brackish H_2O	3/5	3/5	4/5	4/5
Sea H_2O: 2.1‰	2/5	2/5	4/5	4/5
8.5‰	2/3	2/3	4/3	4/3
17.0‰	2/2	2/2	3/2	3/2
35.0‰	-/0	-/0	-/0	-/0

*Pillai and O'Loughlin, 1972.

**Numerator = zoospore activity (Scale: 0-4)
Denominator = sporangial germination (Scale: 0-5)

Although the sporangial wall of *C. opifexi* increases in thickness under high salinities, the thick wall does not protect the sporangium from desiccation, unlike the thick-walled sporangia of *Coelomomyces* of fresh water mosquitoes (38). Thus, complete drying of pools can lead to elimination of the fungus, but if the soil remains moist, the fungus

may survive for several months in intact larval cadavers.

The general observation is that the fungus, *Dermocystidium marinum*, which produces epizootics in oysters, is absent in all very low salinity areas (less than 10-15 ‰) as well as in a few high salinity areas (greater than 30 ‰), but is prevalent in virutally all moderate to high salinities. Hoese (18) has investigated the possible reason for the absence of this fungus in oysters in Port Aransas, Texas, and believes that an inhibitor which occurs in the water stops development of the fungus hypnospores (resistant spore). The origin of the inhibitor is not known. *D. marinum* is considered to be a pathogen which kills oysters during the warm periods of July through October (2, 3). The author claims that *D. marinum* proliferates readily only at temperatures above 25 C and overwinters in oysters as subclinical cases (2).

Another serious pathogen of oysters is the sporozoan, *Minchinia nelsoni*, which commonly was known as "MSX" prior to its identification. This sporozoan favors high salinity (greater than 15 ‰) and its prevalence in the estuaries may vary with salinity changes (1, 2, 3).

Infection of the protozoan, *Paramoeba perniciosa*, on the blue crab, *Callinectes sapidus*, appears to be limited to high salinity waters (44). High temperatures (30 C and higher) appear to favor the bacterial pathogens (*Vibrio* and *Pseudomonas*) of bivalve larvae (15).

In the case of *Coelomomyces opifexi*, field observations in the winter months have corroborated the laboratory study that this fungus can infect *Aedes australis* larvae when the mean temperature is lower than 7 C (35).

The micrococcus, *Gaffkya homari*, which causes Gaffkemia in American (*Homarus americanus*) and European lobsters (*H. vulgaris*) may live as a free-living organism, since *Gaffkya*-like bacteria are readily isolated from mud samples of tidal pools (46). *G. homari* have been isolated from the mud of tidal pools and also from sea water several miles from infected ponds (42).

BIOTIC FACTORS IN MARINE HABITAT

Pathogens of marine invertebrates are known to have alternate hosts. *Gaffkya homari* causes fatal septicemia not only in the American and European lobsters, but also in other crustaceans, such as shrimps, crabs, etc. (46). "The fact that *G. homari* can infect a variety of hosts, with some of these species able to carry the pathogen for extended periods (80 days or more in *C. irroratus*), coupled with its ability to survive in the absence of a host, gives *G. homari* an extremely broad-based potential for survival" (46). This statement would also apply to other pathogens with alternate hosts and carriers. Strains of marine *Vibrio* sp. are pathogenic to several species of bivalve mollusks, and can be isolated from overtly healthy, diseased or moribund bivalve mollusks or their environments (56, 57).

The fungi, *Atkinsiella dubia* and *Pythium thalassium*, which infect

Pinnotheres pisum, have an extensive host range which includes *Gonoplax*, *Trypton*, *Crangon*, *Leander*, and *Portunus* (26). The flagellate, *Hexamita nelsoni*, is a pathogen of several oyster species (43). The gregarine, *Nematopsis ostreacum*, infects oyster, pecten, and other marine gastropods (43). The microsporidans, *Nosema legeri* and *N. dollfusi*, have several hosts belonging to different species of marine bivalves. Gregarines, coccidians, microsporidans, and haplosporidans have several host species of decapod crustaceans (44).

Although no secondary host of the damaging sporozoan, *Minchinia nelsoni*, is known, the failure of the epizootic caused by this sporozoan to decline, even with the severe reduction of the oyster populations, suggests that another unknown host may be involved (3). With the fungus, *Dermocystidium marinum*, "the decline of *Dermocystidium* with the disappearance of oyster populations strongly implies that in its life cycle the parasite is not dependent for winter survival or transmission upon other hosts or resting stages living outside oysters" (1). Two species of ostracods, which feed on the exoskeleton of moribund mosquito larvae infected with *Coelomomyces opifexi*, expose the sporangia which germinate readily (38). It is believed that in permanent pools the ostracods and other scavengers and predatory organisms, including predatory mosquitoes, may play a crucial role in maintaining a cyclic infection of coelomomycetes.

CONCLUSION

Although data are limited, there is evidence that some pathogens of terrestrial and aquatic insects are capable of persisting in the host habitat, outside of living organisms, for several years. There is a serious need, however, for more studies and data, especially under field conditions. This review reveals that information is scarce or lacking in nearly all of the factors listed in Table 1. Studies should be conducted also on problems associated with the aquatic environment, such as industrial and agricultural pollutions, the eutrophication of lakes, and the tremendous outbreaks of marine organisms, such as the red tide.

Some pathogens of terrestrial insects, i.e., *Bacillus thuringiensis*, *Beauveria bassiana*, and *Metarrhizium anisopliae*, are facultative pathogens and may develop outside their hosts. Since they have been applied to control aquatic insects, especially mosquitoes, their persistence in the aquatic environment should be more fully investigated.

Insect pathogens may persist in the aquatic environment for long periods, but their persistence would not be expected to cause problems similar to those of chemical insecticides, such as DDT, where the chemicals accumulate in various levels of the food chain and increase in quantities, attaining a very high concentration at the topmost trophic level of the predatory animals. In the case of pathogens, their host specificity would restrict their abundance mainly to one trophic level. Moreover, the pathogens would be much more readily degradable than the

chemical insecticides because of the more numerous and varied external forces, living and nonliving, involved in their degradation.

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DISCUSSION

MARSHALL LAIRD: I didn't know what I was supposed to discuss until my arrival here. And that arrival coincided with Hank Aaron's 715th homer which quite unfairly robbed me of the attention that an 8-minute dash between the two furthest points of Atlanta airport really warranted. And thanks to my athletic achievement between flights last night I wasn't really in a condition to concentrate on my topic until after breakfast and we had to wait an hour for that, and then the various speakers during the day have kept the lights off so I haven't been able to write very much. And because Joe Tanada has so competently covered the scattered literature on this subject, I'm going to restrict myself to some hopefully provocative generalizations.

First of all, I want to emphasize how little we know of pathogen persistence in the aquatic environment by reference to an old favorite of mine, *Coelomomyces*, once thought confined to mosquitoes. It's now known from some other *Nematocera*, from blackflies and from chironomids, and it's known from *Tabanus*, too. But there's an often overlooked old Russian record that goes almost back to the first description of *Coelomomyces*, and this one concerns an utterly unrelated insect, a Notonectid bug. And Notonectid bugs prey on mosquitoes so the question arises, was that Notonectid infected following eating a mosquito larva? Oddly enough, nobody anywhere seems to have followed this up in any real detail by undertaking really comprehensive surveys of the total animal fauna of even one selected vector habitat in order to see whether, in fact, infections do occur in non-transparent aquatic hosts that are rather less easy to handle than transparent dipterous larvae. I'm really going to address myself to the text "seek and ye shall find." Earlier preachers today have emphasized our still enormous lack of knowledge as regards pathogens of aquatic stages of insect disease vectors and pests. I was particularly struck this morning by Dr. Anthony's tables of our newly acquired knowledge, none of it dating back much more than a decade, on the entomopathogenic viruses of mosquitoes. Of his 15 references, 11 concerned work involving quite a few species of mosquitoes, and seemed to provide a basis for beginning to consider occurrence and incidence of these viruses. However, more thoughtful consideration of his data showed that what they really indicated was the occurrence and incidence of Dr. Harold Chapman and his colleagues. In fact, much of what we know of the distribution of pathogens of vectors relates equally well to the success in obtaining research support and travel grants on the part of Dr. Couch and such other long-term enthusiasts as Dr. Weiser, Mr. Muskrat, Dr. Vago, and so on. If we have to say this for mosquitoes and other vector pathogens we're in much greater ignorance as regards pathogens of non-target aquatic organisms associated with mosquito larvae in the aquatic environment. While we know that some *Nosema* of lepidopterans have several hosts, and that between order infections aren't common, nothing has yet been reported on, for example, whether the microsporidans of blackfly larvae are able to parasitize their arthropod predators. And what about their vertebrate predators, since

many fish are commonly parasitized by microsporidans of genera parasitizing aquatic insects? We still don't pay sufficient attention to the stabilization and standardization of terms, the use of which extend beyond frontiers. I have the greatest respect for the fine work of the early 19th century American entomologist Say, but like all non-Americans I don't accept his name "quinquefasciatus" which many of those who have spoken earlier still quite wrongly apply to *Culex pipiens fatigans*. Thus, I tend to divide my American friends into good Americans and bad Americans, and the good Americans who use "fatigans" are still greatly outnumbered by the ones continuing to use "quinquefasciatus." I'd suggest that perhaps it is a little more important that we take more care about using the same name. Especially when the particular case of a cosmopolitan insect which in many parts of the tropics is of supreme importance as the major vector of bancrofti and filariasis. Workers in a number of countries in different parts of the world would have difficulty in recognizing the relevance to their problem of a *Stempellia* which seems to have been described from something called "quinquefasciatus" in the United States of America.

I've really used that argument over what's in a name as a red herring because if we don't care enough to be tidy about standardizing in this respect, one wonders whether we're really not a very long way away from solving the very much more complex and demanding problems of what constitutes specific criteria in pathogens, whether those pathogens are look-alike viruses that may well have distinctive morphological criteria beyond our present capabilities to detect, or microsporidans like Dr. Maddox's that seem to move from genus to genus with temperature. That one smacks of Lysenkoism, I must say. Without a very much better appreciation of the pathogen texts that are found on the various organisms of complete aquatic ecosystems selected for individual study, we are not in a position to do more than venture educated guesses on questions of host specificity or indeed of the persistence of pathogens of insects in aquatic environments. Among the relevant things we perhaps should be doing are making really purposeful collaborative surveys for, and appraisals of, candidate pathogens of mosquitoes and other aquatic vectors. There is a very effective mechanism (which has been mentioned today) to aid in this connection. I refer to John Briggs' WHO international reference center for diagnosis of diseases of vectors. This seems to me to be a very valuable mechanism which perhaps deserves more support than it's currently getting for rapidly obtaining the kind of information on candidates which we so clearly need. I believe we should also be assembling really exhaustive inventories of the total fauna and flora, and their parasite-pathogen faunules, of individual and representative aquatic habitats selected for intensive study. So far, there isn't a suitable pattern of international collaboration existing in this particular connection.

Finally, it might be observed that none of us and none of our organizations are going to achieve the desired results in this field alone. We're entering an era of integrated control that is immensely more complex than anything ever tackled in applied entomology in the past. It's

my firm conviction we need to call for sustained liaison and collaboration among all interested parties in the relevant governmental, academic and industrial sectors.

R. B. JAQUES: I'd like to discuss the possibility of viruses used against terrestrial insects entering the aquatic environment. In this I'm going to talk about the nuclear polyhedrosis virus (NPV) of the cabbage looper and the granulosis virus (GV) of the cabbage worm. These are nuclear inclusion viruses, of the types which we are considering for use in insect control. I'd like to emphasize a point that Dr. Tanada made, namely that the GV and NPV remain active stored in water for long periods. Here we are talking about distilled water, I believe. Naked virions, on the other hand--virions taken out of the inclusion bodies--don't survive long. Here, it is interesting that proteolytic bacteria, found in the bottom of ponds, etc., have very little effect in decomposing the polyhedral protein. Stronger acids and weak alkalis, of course, do dissolve proteins. However, I think it might be quite safe to assume that inclusion viruses could persist in fresh water, and I don't know anything about marine water.

I concluded that viruses and other pathogens as well reach the aquatic environment by three paths. The natural one is applying viruses directly to the water surface. The second path would be application of viruses to trees or crops growing in or near water that may reach the water. The third path, of course, would be infected insects dying in locations from which cadavers could reach the aquatic environment. I wish to discuss briefly why I doubt that pathogens applied to crops or trees would reach the aquatic environment by other means. We know that viruses are inactivated quickly by exposure to sunlight and so forth, and over 50% of the activity is lost in 2 days. If this is true, the virus is not going to survive long after we apply it. Another consideration, of course, is that insects feeding on the foliage would die there and the leaves might fall and end up in streams, eventually in ponds and so on. However, virus within the cadavers does not inactivate as quickly as this. Another point in relation to inactivation or loss of virus activity on foliage is that viruses are not washed off appreciably. For example, several years ago I put some deposits of virus on cabbage leaves and put them in a photoprint washer for about 4 days, and I only lost about 25% of the original activity.

In regard to inactivation of virus in the aquatic environment, how far does UV light penetrate into water, particularly into murky water, as you would find in a pond?

Work done in 1964 showed that in regard to persistence of virus in streams, it is significant that nuclear polyhedrosis and granulosis viruses remain active in soil for long periods. A nuclear polyhedrosis virus of cabbage looper remained active for 318 weeks. There was still a fair amount of activity after that length of time. Virus accumulated in the environment over a period of time, and these viruses, once put in the environment, do persist. Concerning the persistence of these

viruses in soil, are they going to get into the ground water, etc.? There is little leaching of virus from the top layer of soil down into the lower levels. It is interesting, in experiments carried on by myself and Dr. David in England, that when we pass immense amounts of water, through columns of soil on which we'd put virus on the surface, there is little virus percolating through the column of soil. Therefore, we would assume viruses do not leach from the soil. In clear water or in suspensions of soil, it's rather interesting that NPV and GV remain in suspension for about 2 weeks or more. Therefore, in a stagnant pool or pond the virus would remain in suspension. Dr. Anthony and Dr. Clark mentioned that NPV of *Aedes sollicitans* accumulated or at least persisted in the aquatic environment between epizootics. But if the pond dried up, the virus was lost. I was wondering whether the virus was actually inactivated or had settled to the bottom, became adsorbed onto the soil particles and remained there, and when the water came back to the pond it would not be on the surface.

H. C. CHAPMAN: Our Gulf Coast mosquito research lab has some data on the persistence of various species of fungi, the *Coelomomyces*, in specific ponds over a number of years. *Coelomomyces punctatus* has persisted in larval populations of *Anopheles crucians* in one particular site for 8 years. Both *Coelomomyces dodgei* and *Anopheles crucians* in two particular ponds and *Coelomomyces pentangulatus* and *Culex peccator* have persisted in these sites for over six years. We first found the mermithid nematode, *Rhizomermis nielseni*, in larva of *Uranotaenia sapphirina* in 1965 in one particular pond, around Lake Charles, Louisiana. Seventy-one percent of the larvae were infected in collections from this site last week, nine years later. We have data showing persistence of this nematode in mosquitoes in one other pond, for over 8 years. *Myxomermis petersoni*, another mermithid nematode that happens to be specific to *Anopheles*, has been found in larvae in a cypress swamp pond, since 1967, though infection levels have generally been quite low. This nematode also has occurred in another swampy site for a period of 6 years with rather high levels of infection. One hundred percent of a collection of anophele larvae made last week showed multiple infection. This was 6 years after we had initially found these pathogens in this area. Such persistence of species of *Coelomomyces* and nematodes is rather remarkable, considering our rather frequent droughts and the absence of hosts for long periods. A number of years ago we made up a very simple plastic container with an 80-mesh plastic screen in the sides and bottom. This made a good surveillance tool for studying persistence of pathogens in nature. With this container and host larvae from the lab, we've been able to determine the presence of various species of *Coelomomyces*, mermithid nematodes, occluded viruses, and also some microsporidans. This is a good way to check for pathogen persistence in treated areas where the hosts do not occur.

WILLIAM UPHOLT: In general, with predators and parasites used for biological control it is fairly common that you can find some place in the

world where that particular parasite or predator actually keeps the host organism so well under control that the host organism is, for practical purposes, not a pest. The only suggestion of this that I've heard today in the case of pathogens is in the last comments, by Dr. Chapman.

IGNOFFO: *Bacillus popilliae* is an example of an organism which gives long-term control. There was a pine sawfly introduced into Canada and areas of the northern United States in which the virus associated with that was also introduced, and that has kept that species in check, in balance. There is another record of a cabbage looper virus, an isolate from the United States, that was introduced into Colombia about 3 years ago which has in the third year been successfully controlled. In one localized field they took the virus from this and distributed it over large areas, over an entire valley. For the first two years it significantly held the populations down. I'm sure this approach can be utilized and I think Dr. Roberts mentioned that somewhat in some of his presentation. I wouldn't be surprised if we went back and looked at our introduced pests (some of which are pests now and some of which are no longer pests) and may find diseases associated with these introduced pests, which have reduced the population below that constituting a serious economic threat.

TANADA: I would like to enlarge on this aspect. In almost every study which has been made on an insect population, disease plays some role in regulating the insect population. With some pathogens, the insect population is maintained at a very low level, with others at a fairly high level. This is also associated with the dispersion of the pathogens. If a pathogen has a wide dispersal, it may maintain the insect population at a low level.

USE OF MICROORGANISMS TO CONTROL AQUATIC PESTS OTHER THAN INSECTS

Thomas C. Cheng*

ABSTRACT

Use of microorganisms for the control of aquatic pests other than insects is in its infancy, with the only known field trial based on a highly dubious premise that the bacterium under consideration was pathogenic. Specifically, it was claimed that *Bacillus pinottii*, a Gram-variable species, could be used to control *Biomphalaria glabrata*, the major transmitter of the human blood fluke *Schistosoma mansoni*; however, critically conducted laboratory tests have revealed that this bacterium is nonpathogenic.

Except for molluscs, practically nothing is known about possible use of microorganisms in biological control. What little has been done is directed almost exclusively to those species responsible for transmission of disease-causing helminth parasites. Microorganisms suggested include bacteria, fungi, and protozoa, with at least one nematode, *Daubaylia potomaca*, reported as a possible candidate. Unfortunately, none of these potential biological control agents currently are in culture.

We recently have isolated four species of bacteria from the desert snail *Theba pisana* originating in Israel. Epizootiologic evidence suggests that at least one bacterium is a latent pathogen, i.e., it will kill the host but only under lowered ambient temperature. This evidence, coupled with earlier studies on the flagellate *Hexamita nelsoni* in the oyster *Crassostrea virginica*, emphasizes the need for increased information on the internal defense mechanisms of molluscs before a rational search for candidate microorganisms can be initiated. Specifically, we must know if the phagocytes of molluscs and other invertebrates are chemotactically attracted to invading organisms, what induces phagocytosis, and what is the fate of the phagocytized organism? Such information is essential since an effective biological control agent should not become phagocytized, but if it does, it must not be degraded intracellularly. Information pertaining to this systematic approach to developing potentially useful biocontrol agents for aquatic invertebrates other than insects is reviewed.

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INTRODUCTION

Use of microorganisms as biological control agents of aquatic pests other than insects has gained popularity during the last decade; however, realization of this approach is still futuristic. The major reasons why such research is still in its infancy are (a) there is a lack of economic pressure to develop programs with this objective; (b) the traditional type of undergraduate and graduate training being offered in the biological and agricultural sciences has not stimulated students toward this endeavor; and (c) although a rational approach has been developed and followed for development of biocontrol agents for destructive insects (37), a comparable program has not been developed for noninsect invertebrates.

LACK OF ECONOMIC PRESSURE

Certain insects are well known as agricultural pests, vectors of pathogens to man, animals, and plants, and as undesirable molesters of humans in such recreational areas as beaches, camp sites, and parks. Consequently, the economic implications of their presence are vivid and have been translated into monetary losses. On the other hand, although a number of invertebrates are known to be noxious and undesirable, the full impact of their presence usually is not felt in those countries which can afford to support research leading to their eradication. Certain species of non-insect, aquatic invertebrates are associated with seasonal problems in certain restricted areas, for example, the occurrence of "swimmer's itch" or cercarial dermatitis in both fresh and salt water areas of North America where recreation is an attraction. Cercarial dermatitis, however, is a seasonal problem and the resulting economic loss is difficult to document. Furthermore, chemical eradication, rather than biological control, is practiced. Why, then, should agencies sponsor a relatively long-term research program to control the aquatic snails responsible for the emission of the dermatitis-causing cercariae?

Let us examine another example. The occurrence of both gastropod and pelecypod molluscs in reservoirs and drainage ditches can result in blockage of water flow (64, 65). Such problems essentially are "invisible" to the general public and therefore policy makers are not immediately concerned; thus support for research leading to new methods of eradication is usually not available. Where the situation is sufficiently severe to warrant attention, traditional methods involving chemical control are applied with satisfactory results, hence there are those who question the feasibility of biological control.

It is well known that numerous species of aquatic molluscs serve as intermediate hosts for helminth parasites of man and animals. Further, it is agreed generally that the most effective method of disrupting the life cycles of these parasites is through eradication of the molluscan vectors. With between 200 and 300 million cases of human schistosomiasis

in the world (7), there should be sufficient economic pressure to cause national and international health agencies to support research on biological control of the gastropod vectors. Unfortunately, this is not the situation. Human schistosomiasis is caused primarily by three species of trematodes of the genus *Schistosoma*, namely, *S. mansoni*, *S. haematobium*, and *S. japonicum*. Like almost all parasitic diseases, schistosomiasis, although debilitating and at times lethal, is a chronic rather than an acute disease. Consequently, although its role as a public health problem is generally recognized, it is extremely difficult to assess its economic impact. Furthermore, even if an assessment is made (72), the parameters employed are faulty and the correlations and implications open to question. Thus, without the expression of schistosomiasis in monetary terms, it is difficult to convince agencies to support research in chemical molluscicides, let alone biological control.

A similar situation is true for a number of other human helminthic diseases such as clonorchiasis, paragonimiasis, and heterophyidiasis, all involving aquatic molluscan intermediate hosts. These serious parasitic diseases are endemic to developing countries whose limited budgets cannot fund the necessary research. Those interested in control of animal fascioliasis are more apt to gain support than those concerned with human helminthic diseases. This is due to the fact that the loss to the cattle and sheep industries, attributed to fascioliasis, can be readily measured in dollars. Cattle and sheep owners have considerably more political clout than the poor of underdeveloped nations who are the primary victims of other helminthic diseases.

LACK OF EDUCATIONAL IMPACT

In American universities the "core" of each institution is usually the College of Arts and Sciences and the discipline of biology is usually housed within this administrative unit. Such colleges generally teach biology in the Oxbridge or the Germanic or Continental European tradition, i.e., great prestige is heaped upon esoteric research. In recent years, trends of molecular and cell biology have swept the academic scene. Although these aspects of biology are valuable, they also tend to breed a narrow viewpoint. As a result, invertebrate pathology, as far as I can determine, is only offered at one American university. Of course, because of the mission of Colleges of Agriculture, insect pathology is taught formally at some of the land grant colleges.

There is no need to attempt to justify or dignify such disciplines as invertebrate pathology or parasitology. This has been done in a recent publication (6). However, development of biological control agents for aquatic pests other than insects can be equally as sophisticated and challenging as any of the more esoteric areas of biology. But it will take more than a "popularity contest" to put invertebrate pathology into the mainstream of biology. To establish a lasting mark, those of us engaged in research in this area must practice carefully designed and meticulously conducted scholarship. It is only when this becomes the rule that our discipline will be regarded as respectable.

RATIONAL APPROACH TO DEVELOPMENT OF AGENTS

The development of successful biological control agents should be based on understanding: (a) mode of entry, (b) chemotaxis, (c) recognition of self from nonself, (d) intracellular degradation, and (e) mechanisms of pathogenicity.

Mode of Entry

If the target organism is an aquatic invertebrate, entry of the pathogen can only be effected via the oral, body surface, and anal routes. Of course, in the case of poriferans, cnidarians, ctenophorans, and platyhelminths the last mentioned route is not available.

Oral Route.--The feeding habits of non-insect aquatic invertebrates have been studied intensively, and comprehensive reviews are available (25, 36).

The feeding response of cnidarians, which include a number of toxic species, has been the subject of considerable investigation (47). During feeding, most cnidarians first capture and pierce their prey with their nematocysts. Subsequently, a substance escaping from the nematocyst wounds causes the tentacles to contract toward the mouth and the mouth to open. Finally, upon making contact with the mouth, the food is ingested. In view of this series of feeding reactions, it would appear that a microorganism for biocontrol to be introduced via the oral route must be accompanied by the molecule(s) that will cause the host's mouth to open. Some information is available on activators of the feeding response. Loomis (51) has demonstrated that the reduced form of the tripeptide glutathione is the activator for *Hydra littoralis*, Fulton (29) has shown that the amino acid proline is the activator for *Cordylophora lacustris*, Pardy and Lenhoff (59) have demonstrated that the marine hydroid *Pennaria tiarella* gives a feeding response to proline at concentrations as low as 10^{-6} M and that the proline analog pipecolic acid also serves as an activator. Lindstet et al. (50) have shown that the sea anemone *Boloceroides* sp. is stimulated to feed by valine. Since the response to valine is inhibited by isoleucine, and not leucine, it would appear that *Boloceroides* responds to the amino-*n*-butyric acid moiety with a branch point at the β carbon.

The presence of certain ions in the aqueous environment is known to affect activators. For example, it has been shown that ionic calcium must be present for the feeding response to glutathione to occur (48), whereas Lenhoff (47) has reported that magnesium and sodium competitively inhibit the feeding response. Magnesium ions also inhibit the feeding responses of *Anemonia sulcata* (57) and the proline-stimulated feeding response of *Cordylophora* (29). Among other substances, chelating agents, such as EDTA, will also inhibit feeding of cnidarians; however, this inhibition can be completely reversed by addition of calcium ions and to some extent by strontrium ions (48). Potassium ions will decrease the maximum response of hydra to glutathione (47). The role of activators

and inhibitors of the feeding of cnidarians is important if biological control agents are to be introduced into these aquatic invertebrates via the oral route. Attention must be given to providing an appropriate activator to stimulate ingestion. Furthermore, the presence or absence of certain ions and molecules may dramatically alter the feeding response. This is true also of temperature (45) and perhaps other parameters. Fresh-water cnidarians are not usually considered pests; however, a number of marine species, especially the Portuguese man-of-war, *Physalia physalia*, is an extremely toxic pest. For a review of species of cnidarians known to be toxic to man and animals, see Cleland and Southcott (11) and Halstead (30).

Considerably less is known about the feeding behavior of aquatic platyhelminths, although certain species of turbellarians are said to be venomous (31). The major problem concerning flatworms is cercarial dermatitis and schistosomiasis, both of which are contracted when free-swimming cercariae invade the host's skin. The biological control of cercariae has not been attempted or even investigated, although it is known that hyperparasitism of larval trematodes by microsporidans will cause abnormally developed cercariae (12, 13). However, since these cercariae were situated within molluscs, it remains undetermined how the microsporidans enter the cercariae, although the oral route is suspected.

Nothing is known of the factors that control or influence feeding of the aschelminths and nemertines that occur in the aquatic environment, although certain species of infective larval nematodes that temporarily occur in water certainly are health hazards to man and domestic animals. Cheng and Alicata (8) have reported that the third-stage larva of the meningoencephalitis-causing nematode of the Pacific basin, *Angiostrongylus cantonensis*, could be transmitted via the water route. Whether the control of such larvae is feasible is moot.

It is known that certain species of marine annelids are toxic (11, 30, 58), however, no attempts have been made to control them chemically or biologically. Consequently, nothing is known relative to the oral route of infection among these aquatic invertebrates.

Molluscs have been the principal focus from the standpoint of control, an interest stemming almost exclusively from attention to control of schistosomiasis. Furthermore, although biological control has been tried periodically on a model scale, the primary approach is still chemical (7). As reviewed in a later section, several species of bacteria, fungi, and protozoa have been reported as potentially useful biocontrol agents, but the available information does not indicate the route of entry. However, in the case of the microsporidan *Steinhausia* (=*Coccospora*) *brachynema*, Richards and Sheffield (61) reported that the parasite occurs primarily in the intestinal epithelial cells of *Biomphalaria glabrata*, suggesting that its transmission is probably by ingestion. Consequently, bait formulations should be given special attention as they have with chemical control agents.

A number of species of marine echinoderms are known to be toxic (11,

31), but the only major echinoderm eradication program is that against the crown of thorns starfish, *Acanthaster planci*, in the Pacific (see Branham [3], for review). The eradication was effected through direct human efforts, rather than by biological or chemical means. It has been reported, however, that a few species of animals, such as the fishes *Abudefduf curacao*, *Pseudobaliste flavimarginatus* and *Chellinus undulatus*; the triton shells, *Charonia tritonses* and *Cymatium lotorium*; the helmet shell, *Cassis cornuta*; and the painted prawn, *Hymenocera elegans*, may serve, to a very limited extent, as biological control agents because of their predatory habits. No microorganisms have been proposed as suitable control agents. There is some information on the feeding mechanisms of echinoderms (23, 28), although the implications of this basic information from the standpoint of biological control remains undetermined.

Body Surface Route.--The composition of the body surfaces of practically all groups of aquatic invertebrates is known to some extent (25, 36). The importance of such surfaces from the standpoint of entry by microorganisms remains unexamined. With aquatic gastropods, Michelson (55) was able to infect 4 out of 20 *Helisoma trivolvis*, 3 out of 5 *H. trivolvis fallax*, 2 out of 5 *Biomphalaria glabrata*, and 3 out of 5 *B. pfeifferi* with an acid-fast bacillus by stabbing each snail with a contaminated needle. Furthermore, 12 out of 20 *B. glabrata* and 4 out of 10 *B. pfeifferi* were infected with the same bacterium by inoculating the snails. Although these studies were experimental and may not reflect what occurs in nature, it is of interest to note that the bacterium was introduced through the body surface. In natural infection of gastropods by trematode miracidia, one of the most common methods of entry is through the epidermis. Such entry occurs with schistosome miracidia and their molluscan hosts. The body surfaces of molluscs, and possibly other groups of non-insect aquatic invertebrates, are known to serve as routes for infection. Only additional, specially designed experiments will reveal the feasibility of introducing biocontrol agents through the body surface.

Anal Route.--Nothing is known relative to the importance of this route for the entry of pathogens; however, the possibility should not be overlooked.

Chemotaxis

Theoretically, if a species of bacterium is to be effective as a biological control agent, upon entry, it should not be immobilized by the internal cellular reactions of the target organism. In other words, it should not be phagocytized by the host's hemolymph cells. One of the questions that needs to be raised is whether the phagocytes of the target organism are attracted to the invading organism. From the standpoint of biological control, this is a very important point since chemotactic attraction, at least in some instances, may be considered as a prelude to phagocytosis. Unfortunately, experimental evidence correlat-

ing chemotaxis and subsequent engulfment of the foreign material is currently not available; however, Cheng et al. (10) have shown that chemotaxis between molluscan hemolymph cells and certain foreign materials does occur. Specifically, it has been demonstrated that cells of the American oyster, *Crassostrea virginica*, are attracted to the metacercarial cyst of *Himasthla quissetensis*. Recently in our laboratory we isolated four species of bacteria from the desert snail *Theba pisana* and found them to be lethal to this gastropod when the ambient temperature is lowered (10-18 C). These species of bacteria, particularly a coccus, are recognized as "self" by *T. pisana* and are not phagocytized. In fact, this bacterium is widely dispersed throughout the tissues, especially the muscles, of the snail (Fig. 1). The working hypothesis at this time is that this is a case of a latent lethal infection, which becomes activated when the ambient temperature is lowered, at which time the microorganisms kill the snail. The fact that leucocytosis is not apparent in the vicinity of the bacteria suggests that the host's cells are not chemotactically attracted to the bacteria. Consequently, these microorganisms theoretically could be considered as a potentially useful biological control agent.

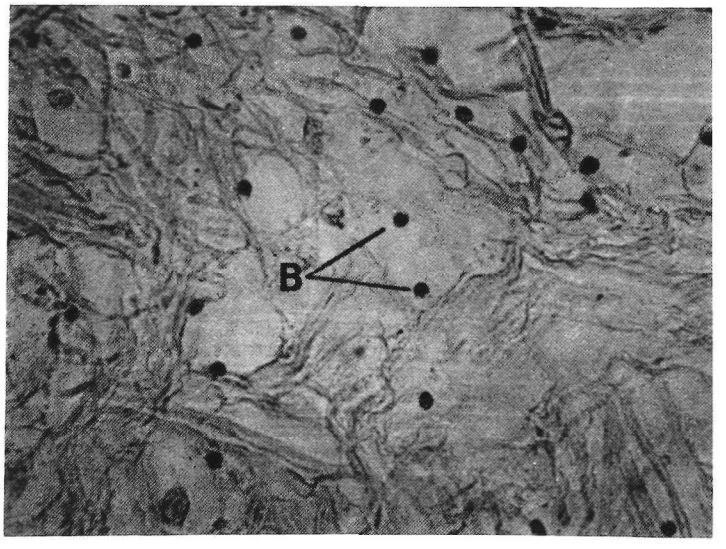

FIGURE 1. Photomicrograph showing nonphagocytized bacterial (B) intermingled among myofibers of *Theba pisana*. (Brown and Brenn stain)

Recognition of Self from Nonself

It appears reasonable that if positive chemotaxis occurs, engulfment of the foreign agent by phagocytosis should follow, but this need not be the case. Chemotaxis and phagocytosis are two different phenomena controlled by different mechanisms. Phagocytosis is essentially a manifestation of the cell's ability to recognize self from nonself.

However, exceptions do exist. Michelson (55) has reported that in planorbid snails, acid-fast bacteria can multiply within phagocytes and presumably can be carried in them to uninfected tissues. Pan (56) has reported occurrence of yeast-like organisms in nerve cells and amoebocytes of *Biomphalaria glabrata* that apparently were not destroyed. Nevertheless, to find suitable biocontrol agents, one should search for microorganisms recognized as self by the target organism and hence are not phagocytized. In vitro testing of phagocytosis may be used as a second screening for potential control agents.

Relative to phagocytosis, encouragement must be given to basic hematologic studies on invertebrates. Although the types of hemolymph cells present in insects have been fairly well defined (39, 40), this is not true of other groups of invertebrates. Only recently have the types of hemolymph cells of two common species of molluscs, *Crassostrea virginica* and *Mercenaria mercenaria*, been defined (26, 27). It has been shown that there are three types of cells in the hemolymph of the mollusc *Mercenaria mercenaria*: granulocytes, fibrocytes, and hyalinocytes (27). Furthermore, based on phagocytic index data when the cells are exposed to *Bacillus megaterium*, it is known that the granulocytes are the most actively phagocytic. The ambient temperature influences the rate of phagocytosis, all categories of cells being more actively phagocytic at 22 and 37 C and being essentially non-active at 4 C (Fig. 2). Such studies should be carried out with aquatic molluscs that are the targets of biological control. Those microorganisms potentially useful as control agents should be applied under ambient conditions least favorable to phagocytosis.

Intracellular Degradation

Microorganisms that become phagocytized are usually degraded intracellularly, although exceptions are known. Thus, as a part of a rational approach to the discovery and development of biocontrol agents information must be available on the enzymes present in the phagocytes of the target organisms. Such information should include optimal pH, salt dependency, temperature dependency, and other characteristics of the enzymes. Such information could serve as guidelines for utilization of microorganisms which are not susceptible to the degradation enzymes.

We have studied a number of enzymes in several species of molluscs including *Biomphalaria glabrata*, the major vector for *Schistosoma mansoni*. Table 1 presents findings relative to the specific activities of several lysosomal and other enzymes. These data show that lysozyme occurs in the snail's hemolymph. Consequently, if a bacterium is to be employed for the biological control of this gastropod, it should be a species which will not be affected by the lysozyme.

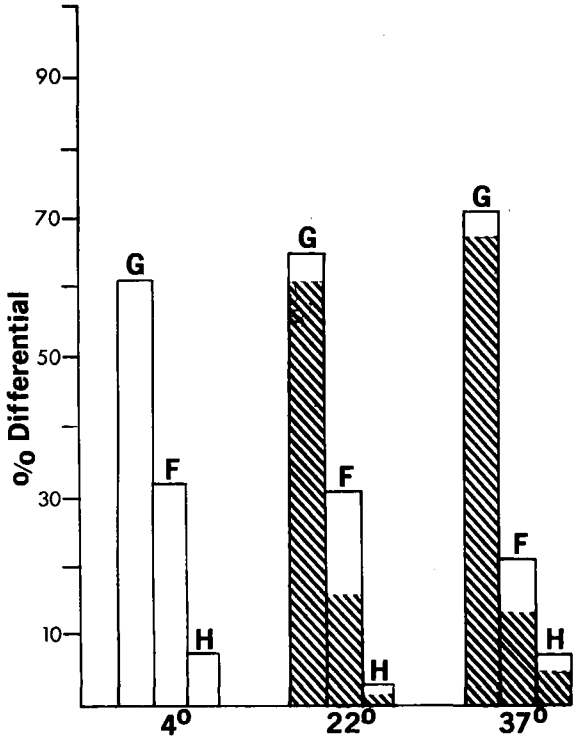

FIGURE 2. Idiograms showing percentage of granulocytes, fibrocytes, and hyalinocytes of *Mercenaria mercenaria* that have phagocytized *Bacillus megaterium* at 4, 22, and 37 C.
G = granulocyte; F = fibrocyte; H = hyalinocyte.

TABLE 1. Specific Activities of Enzymes in Whole Hemolymph of *Biomphalaria glabrata*

Enzyme	Specific Activity	
Alkaline phosphatase	0.5	mμ/mg
Acid phosphatase	2.5	mμ/mg
SGPT (serum glutamic-oxalacetic transaminase)	7.3	Sigma-Frankel μ/mg
SGOT (serum glutamic-pyruvic transaminase)	16.7	Sigma-Frankel μ/mg
β-glucuronidase	29.8	Sigma μ/mg
Amylase	0.13	Somogyi μ/mg
Lipase	0.16	Sigma-Tietz μ/mg
Lysosyme	0.035	ΔOD/mg

Mechanisms of Pathogenicity

Since an effective microbial biological control agent for noxious aquatic invertebrates other than insects has yet to be established, nothing can be said relative to mechanisms of pathogencity. Nevertheless, in view of what is known about viruses, bacteria, and fungi that hold promise as control agents for insects, it is extremely important that the mechanisms responsible for pathogencity be ascertained for microorganisms to be employed for control of molluscs and other aquatic pests.

KNOWN PATHOGENS OF MOLLUSCS

As noted, nothing is known about potential and real microbial control agents for non-insect aquatic invertebrates except for species of molluscs that serve as intermediate hosts for the human-infecting species of schistosomes. This topic has been adequately reviewed by Michelson (54) and Malek and Cheng (53). However, brief accounts of microorganisms suggested as possible biocontrol agents are presented below.

VIRUSES

No virus has yet been identified or isolated from any species of medically important mollusc although virus-like particles have been reported from the octopus, *Octopus vulgaris* (62), and the oyster, *Crassostrea virginica* (22). Successful establishment of cell lines from molluscs has not been achieved, although Cheng and Arndt (9), Hansen and Perez-Mendez (32), and Basch and DiConza (1) have contributed media for maintaining cells of *Biomphalaria glabrata*. The medium of Cheng and Arndt has been employed successfully for transfer of cells through 30 passages. Since viruses are intracellular, obligatory parasites of their hosts' cells, molluscan virology has yet to become a reality.

BACTERIA

A few species of bacteria have been reported from fresh-water gastropods of medical importance. Berry (2) reported a Gram-negative bacterium that caused an epizootic with high mortality in laboratory colonies of *Biomphalaria glabrata*, *B. pfeifferi*, and *Physopsis africana*. This bacterium, unfortunately, has not been maintained in culture. Dias (17, 18, 19) reported a Gram-variable bacterium from the ovotestis of *Biomphalaria glabrata*. This bacterium was originally designated as BET (bacilo de esporo terminal) and later (15) named *Bacillus pinottii*. The organism is saprophytic and develops a high degree of virulence after repeated serial passage in *B. glabrata*. Although Texera and Scorza (69) in Venezuela and Dias and Dawood (20) in Egypt have reported killing *B. glabrata* in the field and in the laboratory with *B. pinottii*, Tripp (70), in carefully controlled laboratory experiments involving *B. pinottii*

from Dias' laboratory, reported that this bacterium apparently is nonpathogenic to *B. glabrata*.

FUNGI

Although no pathogenic fungus has been cultured from a medically important species of mollusc, it is of interest to note that Malek (52) has reported that a species of the Fungi Imperfecti will kill both *Biomphalaria boissyi* and *Bulinus truncatus* in aquaria. In addition, Cowper (14) has observed a species of *Catenaria* invading and destroying the egg masses of *Biomphalaria glabrata* (*Planorbis guadeloupensis*), and Michelson (54) has reported that in a personal communication from De Meillon an unidentified fungus, originating from the hay and grass infusions used to feed the snails, has been observed to invade the tissues of unhatched, embryonic *Physopsis* in the laboratory.

PROTOZOA

A number of ciliates have been reported associated with fresh-water gastropods (16, 38, 41, 44, 71) but none appear to be parasites, or at least pathogens, and hence hold little promise as biological control agents.

Hollande and Chabelard (33) have reported heavy mortalities among laboratory colonies of *Lymnaea*, *Bithynia*, *Biomphalaria*, and *Bulinus* due to a flagellate, *Dimoeriopsis destructor*, and have suggested that this protozoan may hold promise as a biological control agent. Another flagellate, *Cryptobia* (=*Trypanoplasma*) *isidorae*, has been reported from the fresh-water pulmonate *Isidora tropica* (21), but its pathogenicity is unknown. *Cryptobia helicis* is known to occur in the reproductive organ of various species of pulmonate snails including *Triodopsis albolabris*, *T. tridentata*, *Anguispira alternata*, *Helix aspersa*, and *Monadenia fidelis*. This flagellate is apparently nonpathogenic.

Richards (60) reported the occurrence of two species, *Hartmannella biparia* and *H. quadriparia*, in *Bulinus globosus* and *Biomphalaria pallida*, respectively. Because of the general structure of these amoebae, the occurrence of contractile vacuoles (which as a rule are not present in parasitic amoebae), and their sporadic occurrence in aquaria, Richards concluded that these are free-living amoebae that have invaded the gastropods as facultative parasites.

Both *H. biparia* and *H. quadriparia* cause pathologic reactions within their hosts. Specifically, *H. biparia* occur as intracellular parasites within the host's amoebocytes, which, in turn, are surrounded by fibroblasts to form nodules. These nodules, each enclosing several infected amoebocytes, occur throughout the digestive tract, digestive gland, heart, kidney, reproductive system, and mantle. Parasitized snails may become moribund, and the presence of *H. biparia* is believed to affect the growth and reproduction of *Bulinus globosus*.

H. quadriparia also occurs within amoebocytes within nodules, which occur in the foot, tentacles, along the edge of the mantle collar, and in tissues lining the mantle cavity. Infected *Biomphalaria pallida* have been reported to be commonly sluggish and pale and may become moribund. Again, the growth and reproduction of infected snails are believed to be interfered with.

Richards has attempted to infect a number of other species of gastropods through exposure to amoebae. Results are summarized in Table 2. Both species of amoebae could be cultured for several months in medium NCTC 109 diluted tenfold with autoclaved tap water.

Whether these two species of *Hartmannella* are of any use as biological control agents remains to be tested.

TABLE 2. Occurrence of *Hartmannella biparia* and *H. quadriparia* in various species of gastropods*

Molluscan Species	*H. biparia***	*H. quadriparia***
Bulinus globosus	L	-
Bulinus forskalii	E	-
Bulinus guernei	L	0
Bulinus jousseaumi	L	0
Bulinus tropicus	L	0
Bulinus truncatus	L	-
Biomphalaria glabrata	L,E	-
Biomphalaria tenagophila	0	-
Biomphalaria pfeifferi	L	-
Biomphalara helophila	E	E
Biomphalaria abstructa	L	E
Biomphalaria pallida	L	L
Biomphalaria riisei	E	0
Biomphalaria straminea	L	E
Drepanotrema simmonsi	-	E
Helisoma sp.	L	-
Indoplanorbis exustus	L	-
Bithynia sp.	E	E
Physa sp.	E	E

*After Richards, 1968.

**L, found infected in laboratory aquaria; E, found infected after experimental exposure; -, experimental infection unsuccessful; 0, experimental infection not attempted.

COMMENTS ON USING MICROSPORIDA

Microsporidans have been advocated as a major group of microorganisms with promise as biological control agents but the available information suggests that this group of parasites may not be effective as control agents for molluscs. Specifically, as pointed out by Cheng et al. (10), although the microsporida are widely distributed as intracellular parasites of invertebrates (42) where they are commonly pathogenic, they are rarely found in molluscs. Even those species that have been reported from molluscs occur as hyperparasites in trematode sporocysts and rediae, e.g., *Nosema echinostomi* in echinostome rediae within *Lymnaea limosa* (4), *Nosema dollfusi* in *Bucephalus cuculus* sporocysts within *Crassostrea virginica* (67), *Perezia helminthorum* in trematode larvae in Malayan snails (5), the unidentified microsporidan in Malayan echinostome rediae (49), the unidentified species reported by Schäller (63) in larval trematodes in *Tropidiscus planorbis*, and *Nosema strigeoideae* in the intramolluscan stages of *Diplostomum flexicaudum* in *Stagnicola emarginata angulata* (34, 35). As hyperparasites, these microsporidans are protected from the phagocytic action of the molluscan hosts' hemolymph cells and most probably the intracellular enzymes. The same holds true for the microsporidans *Chytridiopsis mytilovum* and *C. ovicola* reported from within the ova of *Mytilis edulis* and *Ostrea edulis*, respectively (24, 43, 66). Both *C. mytilovum* and *C. ovicola* have been transferred to the genus *Steinhausia* by Sprague et al. (68). The only known exception at this time is *Coccospora brachynema*, which Sprague et al. (68) also have transferred to the genus *Steinhausia*. This microsporidan is found primarily in the intestinal epithelium of *Biomphalaria glabrata*. Thus, with this exception, it would appear that the microsporidans, although potentially useful as biological control agents against insects, have not been able to overcome the internal defense mechanisms of molluscs and only have been able to survive in this group of invertebrates as hyperparasites.

EPILOGUE

What I have attempted to do in this presentation is to stress the need for goal-oriented fundamental research that will lead to the finding and development of microbial biocontrol agents for non-insect aquatic invertebrates. Only certain essential elements of what has been termed a rational approach to attaining this objective have been cited. A variety of other factors need to be examined and a number of new "tools" need to be developed. Those concerned with microbial agents for the control of insects know that studies aimed at understanding nutritional and other growth requirements of candidate microorganisms are essential. For viral control agents, invertebrate tissue culture must be developed to make viral cultivation possible.

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DISCUSSION

D. G. AHEARN: I'll not try to add comments directly to Dr. Cheng's excellent review on parasites of a variety of phylla. I will supplement by commenting on selected predation as a possible control of blue-green algae in aquatic habitats. At Georgia State University we have been studying the fate of blue-green algal blooms occurring in the eutrophic region of a large fresh-water lake. The blooms occur periodically in an embayment region which is receiving secondarily treated sewage effluents. Dense concentrations of the algal filaments float to the surface and in small coves. At times several square miles of the lake surface have been covered with a thick algal scum. Shortly after this scum appears, it rapidly changes in texture and within 2 to 3 days the surface of the water develops a short-lasting, white scum. The white scum is composed mainly of an algophagous amoeba. The large trophozoites are capable of ingesting 4 to 5 algal trichomes at once. Digestion of the trichome, based on the disappearance of all but the heterocyst from the time of ingestion, is about 2-1/2 minutes.

This large free-living fresh-water amoeba is capable of consuming extremely large numbers of anabaena and more or less dissolving a nuisance bloom within 2 or 3 days. We have been unable to grow the amoeba on bacteria or stock cultures of anabaena which we have obtained from type culture collections. We have had laboratory blooms by feeding the amoebae with anabaena from the lake. We hope to rear this amoeba in the lab to the extent that we are able to seed algal blooms and initiate digestion. Our preliminary report has been published (1974) in Water, Air and Soil Pollution 3:71-80.

R. CHARUDATTAN: I was very much interested in what Dr. Cheng mentioned in the case of snails and the Mycobacterium, the recognition of self vs non-self. I assume that the recognition of self vs non-self in this example and similar examples is based on the presence of common antigens appearing in the bacterium and the host. I was involved in a study similar to this with plant pathogens and higher plants. Perhaps you could devise a simple and efficient serological test to denote the presence or absence of common antigens. I suppose where you do have common antigens you don't have phagocytosis. Where common antigens are absent, the

bacterium is destroyed by the host. Perhaps this would be an efficient method to screen hundreds of organisms in the laboratory. I would like to do this with plants, but the plant system is a little difficult to handle. Invertebrate immune systems are more organized.

CHENG: Searching for common antigens is a good preliminary screening method, I agree, but I'm sure you also recognize that phagocytosis could be due to factors other than common antigens. There is some information in the literature that mucopolysaccharides elicit endotosis by the host cell. The unanswered question at this time is what induces phagocytosis, especially in invertebrates?

A. M. HEIMPEL: I have two points. One is that I don't think Dr. Cheng completely covered the area of possible biological control, and we have this in insect pathology as well, of the diseases of beneficial animals. In shrimp, oysters and crabs there are a few diseases, some of which are bacterial. The possibility of using phages against these should be explored.

The second point is your concern about the number of schools that teach invertebrate pathology. We in insect pathology and invertebrate pathology have a lot of basic research to do for there is a dearth of good information to work from. When insect pathology started in this country about 25 years ago under Ed Steinhaus, there was only about one school offering a course in this topic. It is my opinion that we shouldn't train too many invertebrate pathologists. As Steinhaus said, both pathology fields cross over disciplines. I think it's better if you have individuals that have no knowledge of insect pathology from separate disciplines who enter invertebrate pathology and contribute their specialties. If you look around at the insect pathology laboratories, for example, the one in Beltsville, Maryland, biochemists, entomologists and microbiologists are all contributing to the field.

SAM SINGER: I don't want to belabor the point in terms of *B. pinottii*, but throughout the symposium we've heard of this or that lost culture. In my own work, it's very difficult to maintain active cultures. We were interested in this *B. pinottii* culture and spoke to Dr. William Haynes of the Northern Regional Laboratory of ARS, especially when his and Ruth Gordon's new monograph was published. I asked him for the culture, which he sent along with a letter saying that they had corresponded with the Oswaldo Cruz Institute and had the feeling that the *B. pinottii* sent to the ARS was the wrong culture. Diaz died in the late fifties and the work of Tripp was in 1961. It could very well be that the culture with which he worked was *B. sphaericus*. The point is that there are bacteria, fungi, etc., which are obviously active, otherwise we'd be flooded with all these diseases. Thus, let us not overlook the possibility of these things. When we hear of *Bacillus matthesi* and so forth being "lost," we should be a little more optimistic and hopefully we will find either *B. pinottii* or some organism like it.

S. R. DUTKY: Dr. Cheng mentioned that the lack of phagocytosis is a favorable characteristic of a pathogen. That probably is true but the best pathogens aren't necessarily those that exhibit all the best points. I think it would not be a good idea to eliminate consideration of a pathogen on the basis that it was phagocytized, because many of the "good" pathogens of man are "bad" ones from this standpoint. The occurrence of phagocytosis only increases the number that is required to produce a threshold infection. This does not have anything to do with its efficiency. If it can produce these numbers by multiplying in the host and destroying the phagocytes, then the phagocytes only become carriers of the pathogen to various areas.

CHENG: What you say is quite true, but from our experience with mollusks, if you introduce a bacterium and it becomes phagocytized, almost all become intracellularly degraded.

DUTKY: That's only a question of numbers. If the exposure level is raised by a factor of one decade, then perhaps not all of the introduced bacteria will become degraded.

CHENG: That is, if they multiply within phagocytes and/or are not susceptible to the cells' enzymes.

DUTKY: No, only if the initial exposure gives you a number which will saturate the capacity of the phagocytes to take them up then the phagocytes are overwhelmed. In other words, you may require ten times the dosage in order to get the introduced bacteria to be effective.

CHENG: I am not sure such a phenomenon has been demonstrated in mollusks. However, I would think that it could be demonstrated easily by inter-hemocoelic injections. One could introduce a number of bacteria and the mollusks are able to recover. However, beyond that number they do not recover. Specifically, a possible relationship between the mortality and ineffective phagocytosis should be checked.

MARSHALL LAIRD: Somebody mentioned *Bacillus matthesi* and black flies. As I recollect, what was called *Bacterium matthesi* was actually isolated in 1935 from Guacina, in what's now Tanzania, and this bacterium was said to have been lost. This phrase keeps cropping up about all these lost cultures and I wonder whether sometimes we sound unduly pessimistic in that some of the earlier work in these areas was really so trivial that there never was any attempt to establish a culture in the full sense of the word. Perhaps we should go and look again and do now what wasn't done 20 or 30 or whatever years ago.

IGNOFFO: From what I've heard reported from the review and what little review I've done in this area, I have yet to find a systematic search

that selects a particular pest and then go to see what's there. Obviously, those that will be observed will be the virulent types of pathogens. But, in fact, the ones you might want to use are the debilitative type, which reduce the host population down to a low level and yet survive in the aquatic environment. I think one of the first things to do is select the model, and search for that model. Concurrently, what you can do is utilize those known pathogens that are available. And from what we've heard here, there are some groups that don't have the specificity of other groups. Is it possible that one of these microsporidans might in fact be an excellent pathogen for the snail that transmits schistosomiasis? I'm just suggesting this, actually, as a systematic approach to solving a particular problem. Now if you select the right experimental animal, the basic information you want to elucidate will be forthcoming. A lot of us do not do this. The impact we can make from our findings is not as great as it can be because of our poor selection of the model to begin with.

USE OF PLANT PATHOGENS FOR CONTROL OF AQUATIC WEEDS*

R. Charudattan**

INTRODUCTION

Among natural enemies of plants the most versatile and ubiquitous are pathogenic and antagonistic microorganisms. In this category are fungi, viruses, bacteria, nematodes, mycoplasms and possibly Rickettsiae. Each species of plant is subject to its characteristic diseases (38). That diseases can be utilized to combat unwanted plants is shown conclusively by successes in controlling terrestrial weeds like Northern jointvetch (10), skeleton weed (9, 15), and others (37). Indeed, biological control of water hyacinth with a *Fusarium* disease was contemplated as early as 1932 (1). However, despite the long existence of aquatic weed problems in the United States and abroad, successful control of water weeds using microorganisms remains to be accomplished. The reasons for this are many, among which lack of studies on aquatic plant pathogens is perhaps the most important (40).

Several species of aquatic weeds pose serious problems in Florida. The more significant ones in order of importance are: water hyacinth (*Eichhornia crassipes* [Mart.] Solms.), hydrilla (*Hydrilla verticillata* Royle), alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb.), and Eurasian water milfoil (*Myriophyllum spicatum* L.). Studies at the University of Florida to seek and evaluate plant pathogens and phytotoxic organisms as biocontrols of water weeds were started in 1970. Since then, we have been concerned with all four species mentioned above, with major emphasis on water hyacinth and hydrilla.

Our work has consisted of six major facets: a) establishing a quarantine facility and developing procedures for bringing foreign pathogens of aquatic plants into the United States from various geographic regions; b) developing techniques to study diseases of aquatic plants; c) conducting local and foreign explorations and seeking effective pathogens of the four target weeds; d) screening available pathogens of aquatic weeds to determine their efficiency, host range, and suitability as biocontrols; e) testing on a limited basis two fungal pathogens on water hyacinth in the field; and f) testing and evaluating microbial metabolites toxic to hydrilla. The following general procedure is used for testing and evaluation of pathogens: a) isolation in

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pure culture, identification, and artificial inoculation on weed hosts; b) establishment of pathogenicity and aggressiveness on target hosts; and c) determination of host range from published information and from our own studies. Once a promising pathogen is found, limited field tests are designed in order to determine its performance under natural conditions, and to ascertain possible environmental impacts of its artificial introduction.

REVIEW OF WORK

A detailed review of research conducted at the University of Florida is available (13). The following is a summary of our findings concerning the four target weeds.

SURVEYS

Several local and foreign explorations were undertaken to collect and test pathogens of aquatic weeds. Some pathogens of water hyacinth and alligator weed were found locally (17, 29), although several pathogens reported abroad were not located in the United States. Diseases of hydrilla and Eurasian water milfoil were not encountered in Florida. As these weeds entered this country, their pathogens were not necessarily among them. Once here, and free of their pathogens, such plants may be free of major diseases for long periods of time until original pathogens are introduced (14). Foreign explorations were considered important because these plants are likely to be in balance with their natural enemies in their native habitats. Thus, the probability of finding suitable pathogens is considerably greater in areas from which these plants originated. The following countries were surveyed for plant pathogens: Barbados, Jamaica and Trinidad of the West Indies, the Dominican Republic, El Salvador, Guatemala, India, Mexico, Panama, Puerto Rico and Venezuela.

QUARANTINE PROCEDURE

Introduction of foreign microorganisms into the United States must be approached with utmost caution. The potential dangers to local agriculture and to environment from such introductions must be evaluated before any large-scale use of imported pathogens can be contemplated. For this purpose, a quarantine greenhouse facility was built in Gainesville in which all foreign isolates suspected to be pathogens of the four target weeds were housed with maximum security. The quarantine facility was approved by the U.S. Department of Agriculture and the Florida Department of Agriculture and Consumer Services. To date, about 600 fungal and bacterial isolates and two suspected viruses of foreign origins have been tested on water hyacinth and hydrilla in this greenhouse.

PATHOGENS OF TEST PLANTS

WATER HYACINTH

A list of identified pathogens of water hyacinth and their presence or absence in the United States on water hyacinth and other hosts appears in Table 1. Of these, isolates of *Rhizoctonia solani* Kuehn from India, Panama, and Puerto Rico have been studied intensively (5, 11, 18, 29), and in artificial inoculations was found to be the most aggressive of the pathogens on water hyacinth. *R. solani* induces severe, irregular lesions on leaves, blighting, and often death of plants (Fig. 1). Studies by Joyner (18) indicated that isolates of this fungus from Panama, Florida, and in culture collections in this country varied with regard to virulence, temperature optima for infection, host specialization, and ability to infect emersed versus submersed portions of aquatic plants. All isolates tested were capable of attacking 14 species of aquatic plants of 9 families. According to an early report (4) *Rhizoctonia solani* was responsible for destruction of valuable aquatic plants in Virginia and North Carolina that were a source of food for ducks. In areas where this pathogen was found on water hyacinth, it did not seem to affect other vegetation (29). The potential of this fungus as a biocontrol agent of water hyacinth cannot be ignored (13).

A zonal leaf spot of water hyacinth caused by *Cephalosporium zonatum* Sawada was found in Puerto Rico, Florida, and Louisiana (Fig. 2). A disease with identical symptoms has been ascribed to *C. eichhorniae* Padwick in India (24). The two species, as well as *C. fici* Tims and Olive, the causal agent of leaf spot of fig, are probably synonymous (30). The pathogen is quite virulent and destructive on leaves of water hyacinth, often causing necrosis of most of the leaf area. Greenhouse tests on this fungus indicated a broad host range. Of 17 plants (12 families) tested, 16 were susceptible, including one submersed aquatic plant (30). Despite this wide host range under laboratory conditions, *C. zonatum* attacks naturally only fig (34) in North America. In view of its narrow host range in nature, this pathogen has good potential as a biocontrol agent of water hyacinth.

A leaf blight of water hyacinth caused by *Alternaria eichhorniae*, Nag Raj and Ponnappa, from India, may hold promise as a biocontrol agent (21). This fungus was among those isolated from that country by this investigator. Tests in India indicate it is highly host-specific. Of 41 species (19 families) tested, including 7 aquatic plants, only *Monochoria vaginalis* Pers. of the same family as water hyacinth was susceptible to this pathogen. In addition, *A. eichhorniae* produces a metabolite of pigment origin toxic to water hyacinth (21). Tests with this pathogen in our quarantine facility have been encouraging. It is a virulent pathogen of water hyacinth (5) and may prove useful in Florida as a biocontrol of the plant.

TABLE 1. List of Water Hyacinth Diseases, Causal Agents and Their Presence or Absence in the United States

Disease	Causal agent	Presence or absence in United States	
		On water hyacinth	On other hosts
Blight	*Alternaria eichhorniae*	?	-
Leaf spot	*Apiocarpella* sp.	+	?
Zonal leaf spot	*Cephalosporium zonatum* (= *C. eichhorniae*) (= *C. fici*)	+	+
Leaf spot	*Cercospora piaropi*	+	-
Leaf spot	*Curvularia clavata*	-	-
Leaf spot	*C. lunata*	+	+
Smut	*Doassansia eichhorniae*	-	-
Blight	*Drechslera* sp.**	-	?
Leaf spot	*Fusarium roseum* (= *F. equiseti*)	+	+
Leaf spot	*Helminthosporium bicolor*	-	
Thread blight	*Marasmiellus inoderma*	-	
Leaf spot	*Mycoleptodiscus terrestris***	+	+
Leaf spot	*Myrothecium roridum*	-	+
Leaf spot	*Nigrospora sphaerica*	+	+
Leaf spot	*Pestalotia* sp.	+	+
Leaf spot	*Phoma* sp.**	+	+
Blight	*Rhizoctonia solani* (= *Corticium solani*) (= *Hypochnus sasaki*)	+	+
Blight	*Sigmoidea* sp.**	+	?
Rust	*Uredo eichhorniae*	-	

*Compiled from references 29, 35, 40.

**Charudattan, unpublished data.

A variety of *Myrothecium roridum* Tode ex Fr. from India was reported to be pathogenic to water hyacinth (20). Several isolates of this pathogen were collected and tested in Florida (5). It is highly pathogenic to water hyacinth, but is also known to attack a wide variety of other plants (27). In this country it is a significant pathogen of ornamental plants (35). Further tests on the host range of this organism are continuing. Recently, an isolate of *Drechslera* sp. has been obtained from water hyacinths in the Dominican Republic. In preliminary tests, this organism equaled *R. solani* in its pathogenicity on water hyacinth. Further tests are underway. *Cercospora piaropi* Tharp. was described in 1917 on *Piaropus crassipes* (= *E. crassipes*) from Texas (33). Its occurrence in this country has recently been reconfirmed (12). Since species of *Cercospora* are frequently host

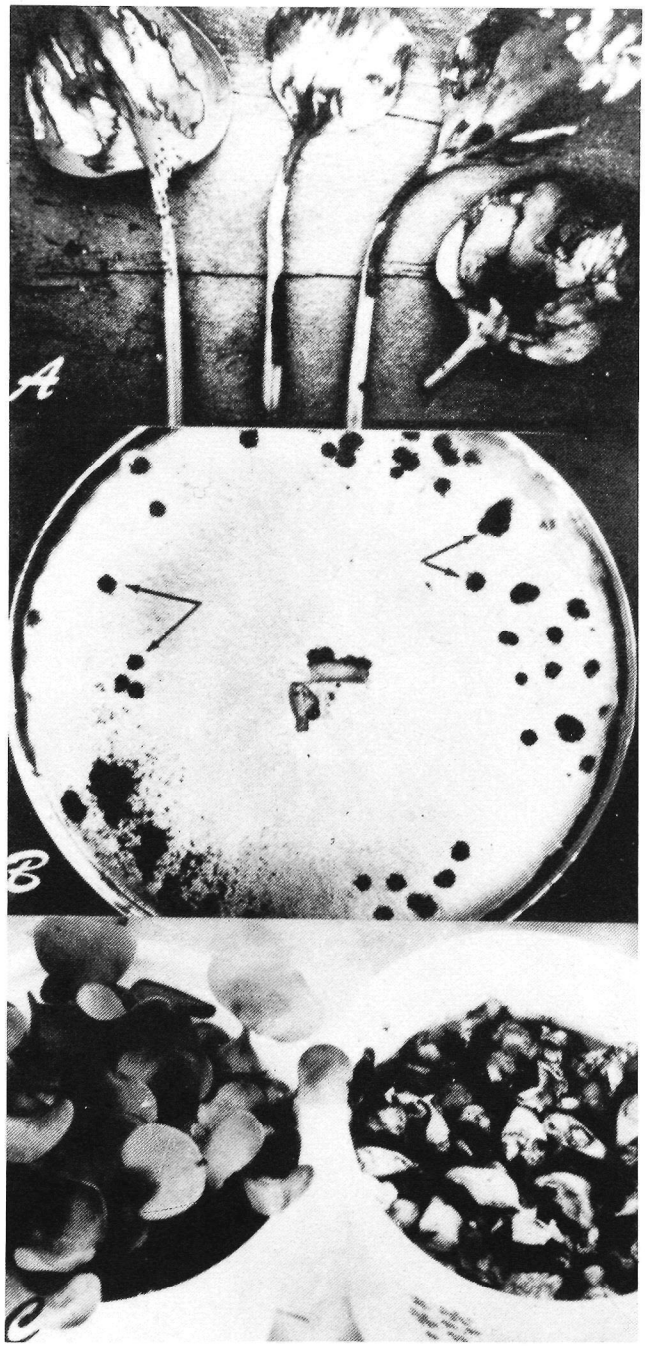

FIGURE 1. Blight of water hyacinth incited by *Rhizoctonia solani*: a) Blighted leaves of anchoring water hyacinth (*Eichhornia crassipes*) from Panama. b) The fungus from leaves A; sclerotia, marked by arrows, can survive in water for over 26 months. c) Dead water hyacinth plants (*E. crassipes*), inoculated with B (right) and noninoculated controls (left). From Rints (29).

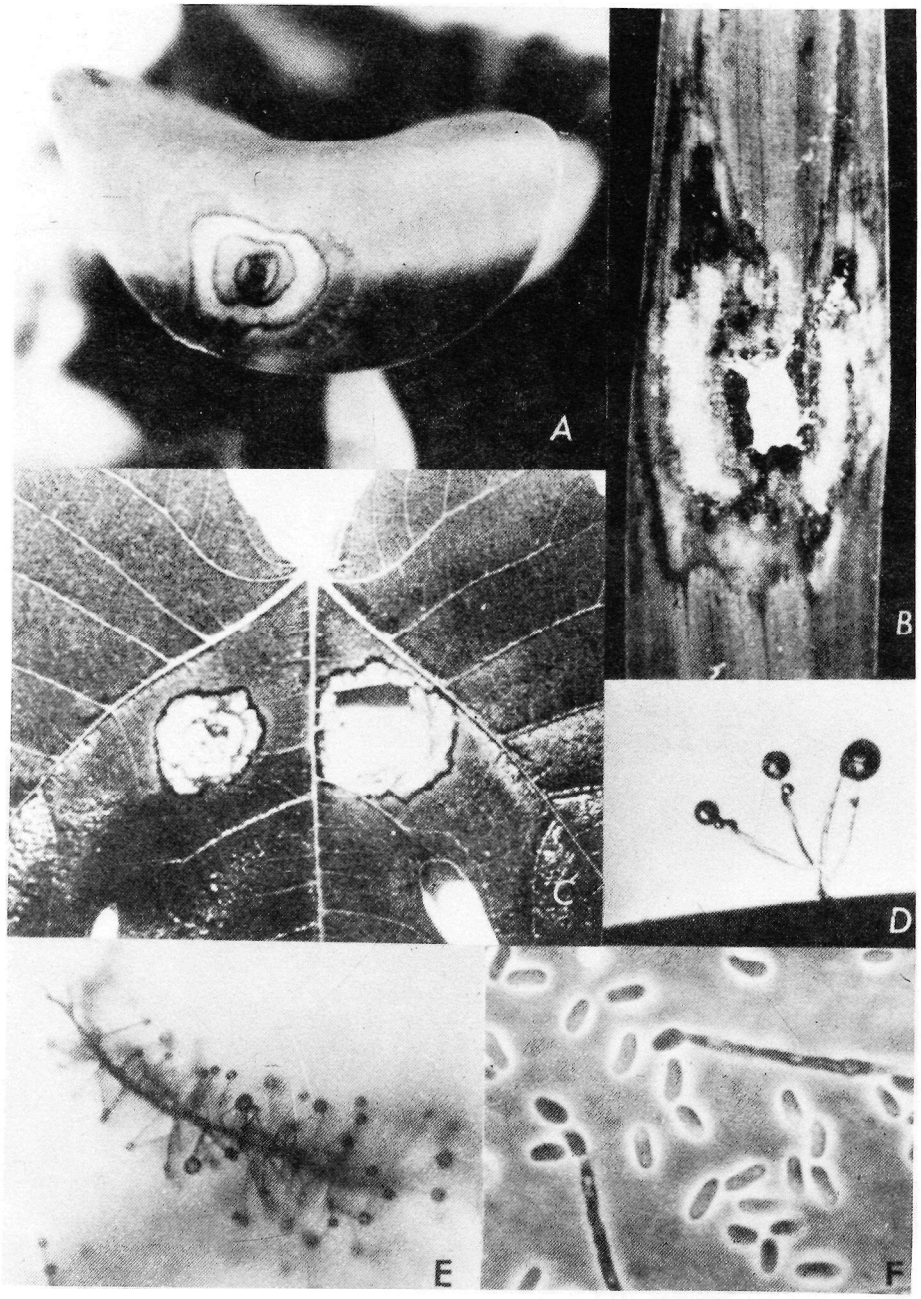

FIGURE 2. Symptoms and morphological characteristics of *Cephalosporium zonatum*: a) Zonate lesion due to *C. zonatum* on water hyacinth leaf. b) Lesion on narrow-leaved pickerel weed (*Pontederia lanceolata*). c) Lesions on a leaf of variety Celeste of fig. d) Single branched conidiophore with spore heads (X360). 3) A specific diagnostic feature: a strand of funiculose hyphae bearing conidiophores and spore heads (X80). f) Conidiophores and conidia (X800). From Rintz (29).

specific, tests are being conducted on the usefulness of this pathogen against water hyacinth. Several other pathogens listed in Table 1 have been tested and maintained in our collection. Their use in control of water hyacinth may be obscure due to their lack of aggressiveness on the plant.

A number of bacterial isolates have been obtained as potential pathogens of water hyacinth. However, none of the isolates tested so far has proven pathogenic. Numerous plant pathogenic viruses were tested on water hyacinth, hydrilla and alligator weed (Table 2), but none infected any of these weeds.

TABLE 2. Plant Viruses Tested on Water Hyacinth, Hydrilla and Alligator Weed*

Virus Group**	Virus	Indicator Plant Species***
Bromovirus	Brome mosaic	*Horedeum vulgare* 'Moore'
Cucumovirus	Cucumber mosaic	*Chenopodium amaranticolor*
Nepovirus	Tobacco ringspot	*Nicotiana tabacum* 'Turkish'
Potexvirus	Clover yellow mosaic	*Pisum sativum* 'Alaska'
	Cymbidium mosaic	*Cassia tora*
	Papaya mosaic	*Gomphrena globosa*
	Potato virus X	*G. globosa*
	Papaya ringspot	*Comphrena globosa*
	Sugarcane mosaic strain E	*Zea mays* var. *saccharata* 'Golden Cross Bantam'
	Tobacco etch	*N. tabacum* 'Turkish'
Tobamovirus	Odontoglossum ringspot	*C. amaranticolor*
	Tobacco mosaic (aucuba strain)	*N. tabacum* 'NN'
	Tobacco mosaic (type strain)	*N. tabacum* 'NN'
Monotypic	Tobacco necrosis	*N. tabacum* 'Turkish'
Other	Barley stripe mosaic	*H. vulgare* 'Moore'
	Bacilliform virus isolated from Southistle	*N. clevelandii* x *N. glutinosa*
	Hippeastrum mosaic	*Hippeastrum* sp.
	Dasheen mosaic	*Philodendron selloum*
	Onion yellow dwarf	*Allium cepa*
	Southern bean mosaic	*Phaseolus vulgaris* 'Kentucky Wonder Wax'
	Wheat streak mosaic	*Tritichum aestivum* 'Georgia 1123'

*F. W. Zettler, unpublished.

**After P. Wildy's Classification and Nomenclature of Viruses, 1971 (Monographs in Virology. S. Kargar, Basel). "Other"--not in Wildy's list.

***Inoculated before and after each trial and also used for back inoculation attempts.

HYDRILLA

Diseases affecting hydrilla were unknown prior to our study. In 1973, two diseases of hydrilla from India, caused by a *Pythium* species and *Sclerotium rolfsii* were found (5). They induced chlorosis, yellowing and lysis of test hydrilla plants (Fig. 3). In addition, about 15 isolates of *Aspergillus*, *Penicillium* and *Trichoderma* that produce metabolites toxic to hydrilla have been found from Florida and India (Table 3). Twelve of these have been reported to produce substances in liquid cultures toxic to hydrilla (6). The toxic culture solutions induced chlorosis of test plants followed by death and lysis of the dead remains of plants (Fig. 4). The effects of toxins were visible within six days after mixing the culture solutions. The toxin of four of the isolates was oxalic acid (6), known to be involved in diseases caused by *Sclerotinia schlerotiorum* (19) and *Sclerotium rolfsii* (3). Since oxalic acid is a potent biotoxin, its potential in controlling hydrilla is considered poor. Studies are underway to identify and determine the nature of other toxins from fungi, hopefully with more specificity than oxalic acid.

TABLE 3. Fungi Pathogenic to Hydrilla*

Fungi	Source
Pythium sp.	India
Sclerotium rolfsii	India, Florida
Chaetomium sp.**	India
Aspergillus spp.	India, Florida
Penicillium spp.	India, Florida
Trichoderma viride	India, Florida

*Charudattan (5).

**Charudattan, unpublished data.

ALLIGATOR WEED

A virus-induced stunt of alligator weed, characterized by an overall stunting of the plant, was found in the Ortega River near Jacksonville in 1971 (17). Affected leaves were smaller than normal, reddish in color, and often distorted. Electron microscopy of affected leaves showed presence of a flexuous rod type virus (Fig. 5). The nature and length (1587-1781 nm) of the virus particles suggested that the causal agent belonged to the beet yellows group of viruses (17). Members in this group are aphid-transmitted. However, laboratory transmission was not accomplished using *Myzus persicae* and *Aphis gossypii* which normally colonize alligator weed. The virus was graft transmissible (Zettler, unpublished), but other methods of mechanical transmission were unsuccessful. The virus persists in plants vegetatively propagated from

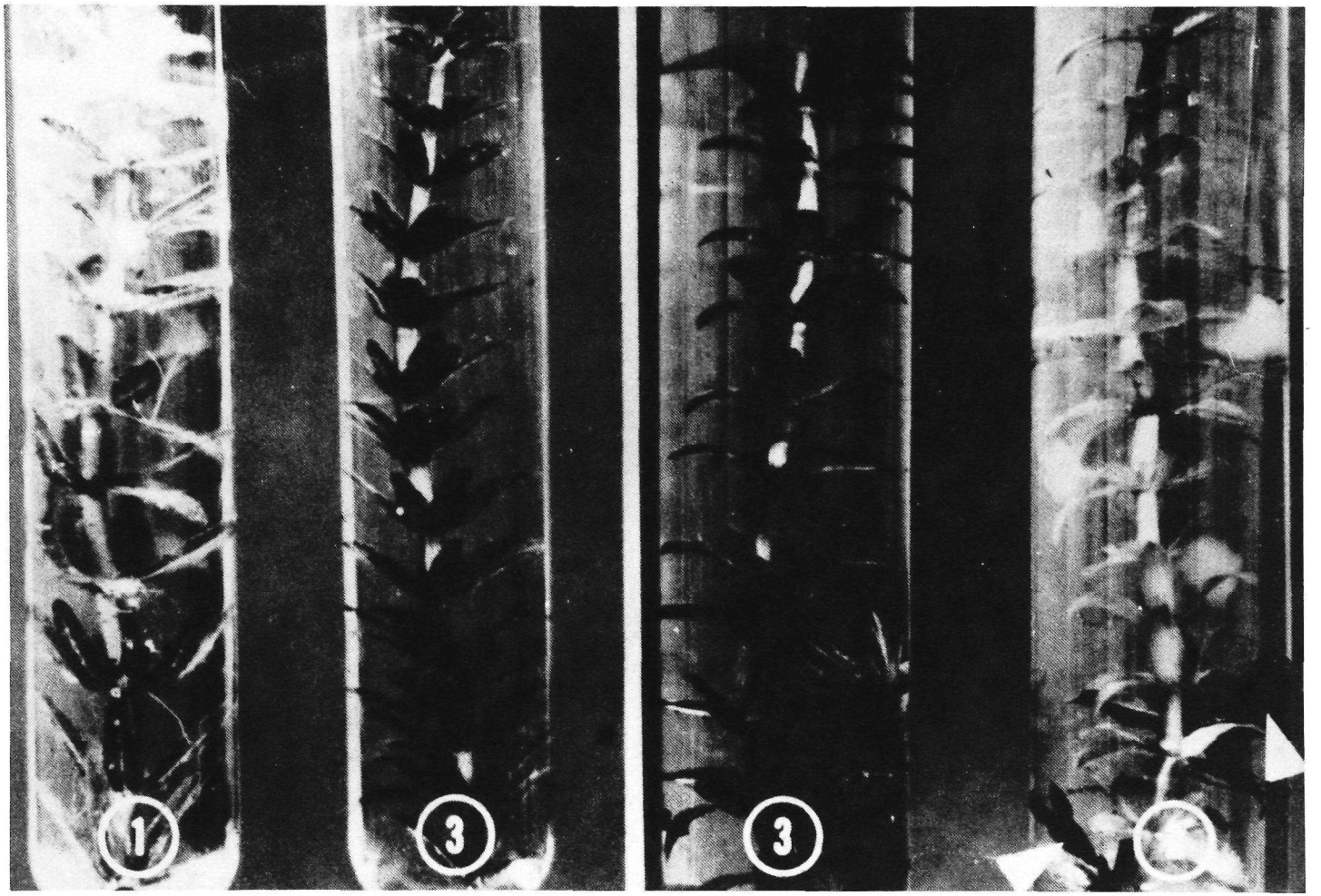

FIGURE 3. Damage to hydrilla caused by *Sclerotium rolfsii* and *Pythium* sp. 1) Hydrilla with discoloration especially of the growing tip induced by *Sclerotium*. 2) Damage due to *Pythium*. Arrows point to development of green axillary shoots. 3) Control. From Charudattan (5).

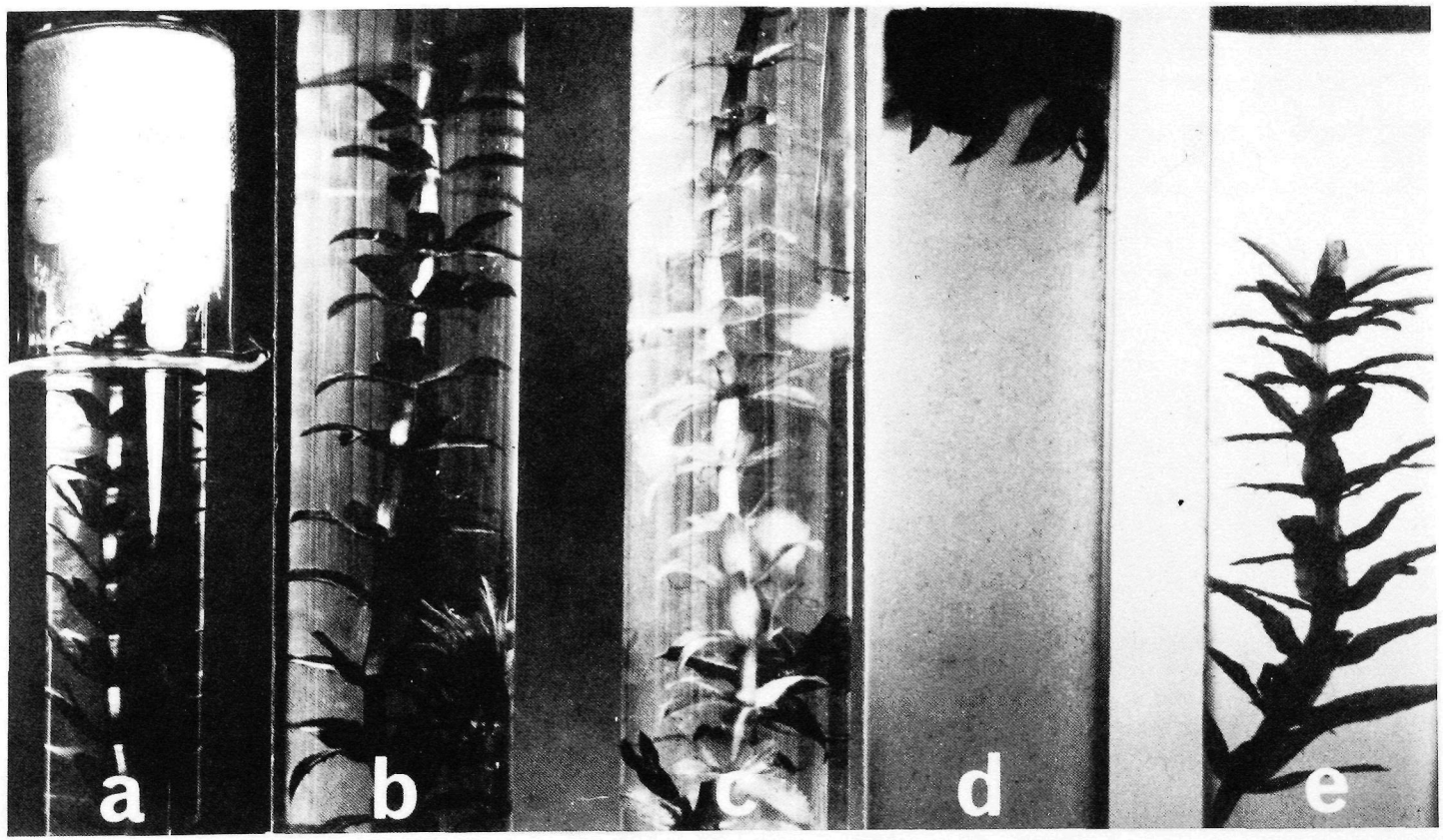

FIGURE 4. Test tube assembly used for testing pathogenicity of organisms to hydrilla (a). Control (b) and *Penicillium*-infected hydrilla (c). Note the pale appearance of hydrilla in (c) due to the fungus. Lysis of hydrilla caused by toxin from *Penicillium* (d) and control plant (3). From Charudattan (5, 6).

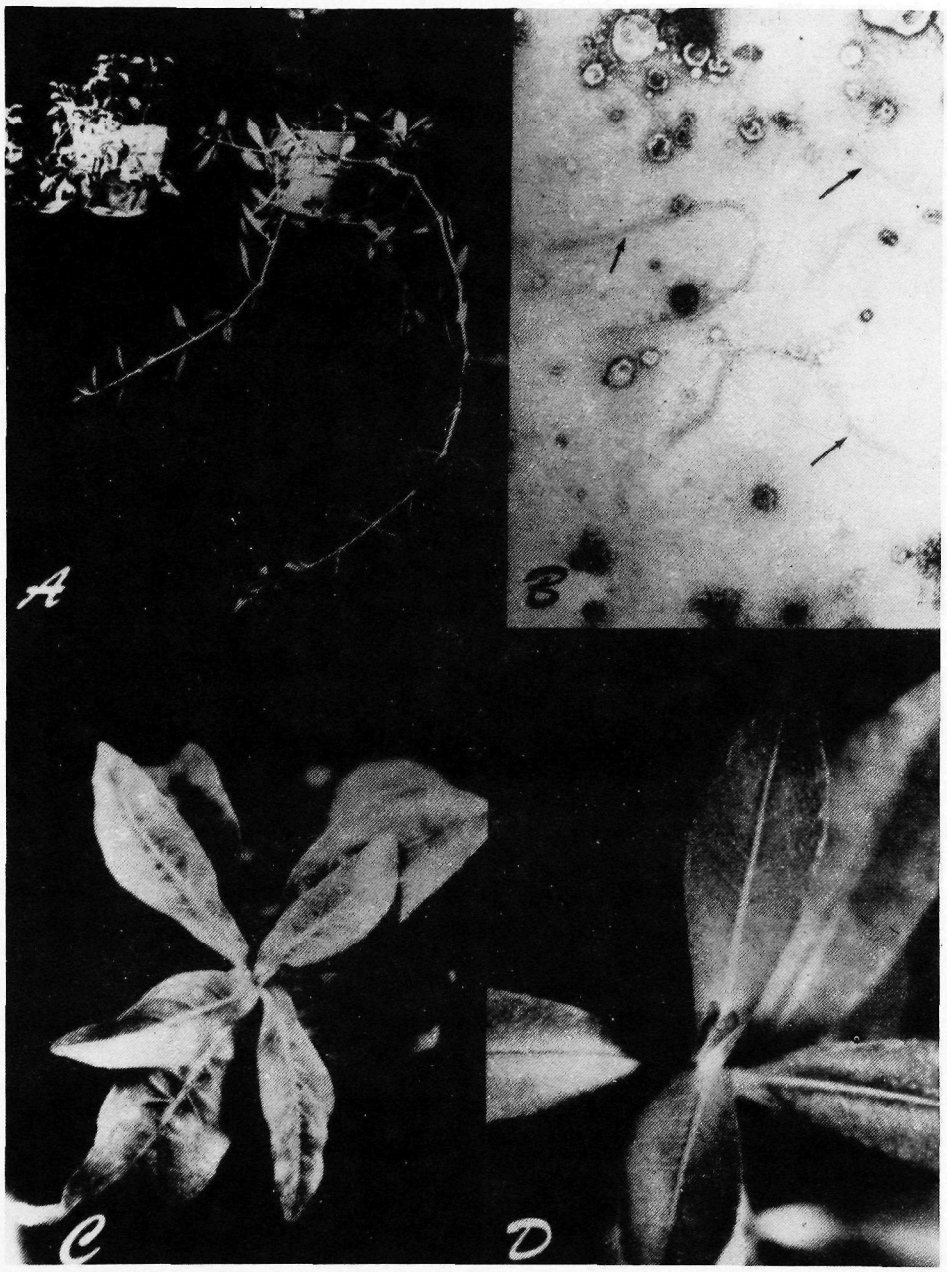

FIGURE 5. Virus stunt of alligator weed. A) Healthy (right) and diseased (left) alligator weed plants; infected plants are markedly stunted and grow at a slower rate than healthy plants. B) Virus particles (arrows) as seen with the electron microscope. C) Magnified view of shoot apex of infected alligator weed plant showing pronounced foliar distortion and twisting as compared with healthy shoot apex of this plant (D) at the same magnification. From Zettler, unpublished.

diseased material, and holds promise for controlling alligator weed. Unfortunately, this virus is not readily transmitted in nature, as repeated searches subsequent to 1971 in the Ortega River failed to disclose its presence. Table 4 includes known pathogens of alligator weed, of which the significant one is the virus.

TABLE 4. Pathogens of Alligator Weed

Pathogen	Occurrence in United States	Reference
Alligator weed stunt virus	+	17
Rhizoctonia solani	+	35
Heterodera marioni	+	40
Anguillulina dihystera	+	40
Uredo nitidula	-	2
Meloidogyne sp.	+	35

EURASIAN WATER MILFOIL

Surveys in Florida for diseases of Eurasian water milfoil did not disclose any pathogen of this plant. Pathogenicities of several fungi from other plant hosts were tested on this plant. Included were four species of *Fusarium*, 11 species of *Pythium* and seven species of *Phytophthora*. The latter two taxa are phycomycetous genera and produce zoospores motile in water. Another phycomycete, *Aphanomyces euteiches*, also was tested in Eurasian water milfoil because of its high degree of virulence on *Echinodorus brevipedicellatus*, a submergent aquarium plant (28). None of these pathogens infected Eurasian water milfoil, however (16). Several bacteria obtained from diseased Eurasian water milfoil plants were non-infective under laboratory inoculations (16). Only a Panamanian isolate of *Rhizoctonia solani* from *Eichhornia azurea* was pathogenic to this plant and *Myriophyllum brasiliense*. The fungus caused local necrosis on submersed portions of the stem and consequent toppling of portions above necrotic zones. Usually healthy side shoots emerged from uninfected portions of the stem. Due to its inability to kill the plant, this isolate of *Rhizoctonia* was not considered a likely biocontrol for Eurasian water milfoil (16).

FIELD TESTS

Limited field tests have been conducted with *R. solani* and *C. zonatum* on water hyacinth by T. E. Freeman (unpublished). None of the other pathogens or toxic isolates are currently being field tested on any of the other three target hosts. The test site for field trials with water hyacinth is in Lake Alice, situated within the boundaries

of the University of Florida campus in Gainesville. The area involved is approximately 30 ha in size and has been infested by water hyacinth for several years. For inoculum, fungi were grown in flasks containing Czapek-Dox broth for 14 days. Mycelia and spores were harvested, homogenized in a blender, and sprayed on water hyacinth plants with a pneumatic sprayer. Pathogens were applied singly and in combination. Plants became readily infected within two weeks, and characteristic symptoms were visible as early as a week with *R. solani* and in two weeks with *C. zonatum*. In two months, secondary spread of *C. zonatum* was apparent. The disease incited by *R. solani* was, however, less than expected. These trials showed that the two organisms can be utilized for artificial induction of diseases on natural populations of water hyacinth. Additional field tests with *C. zonatum* are in progress to determine its pathogenicity to several terrestrial crop plants.

FUTURE PLAN OF WORK

A top priority in our work is to obtain rust and smut pathogens of water weeds reported from South America. Rusts and smuts, theoretically, are the most ideal pathogens to control weeds. Due to their host specificity, virulence, and ability to invade reproductive organs of plants rusts and smuts are likely to be effective as biocontrol agents. *Uredo eichhorniae* Frag. and Cif.--a rust, and *Doassansia eichhorniae* Ciferri--a smut, have been reported on water hyacinth from the Dominican Republic (8, 7). A rust, *U. nitidula* Arth., was discovered on alligator weed from Guatemala (2). However, a recent search in the former country (Charudattan, unpublished) and an earlier survey by Hill and Rintz (unpublished) in the latter did not reveal the presence of any of these three pathogens. Literature on South American rusts is scanty, and personal correspondence with 25 plant pathologists in 13 Central and South American countries did not establish either the presence or absence of these three or other obligate pathogens of target weeds. Currently, attempts are being made to rediscover the above three pathogens, but excessive reliance on them for biocontrol of aquatic weeds may be unwise.

Another current area of intensive investigation is possible use of *C. zonatum* in an integrated control of water hyacinth along with the weevil, *Neochetina eichhorniae*, and the mite, *Orthogalumna terebrantis*. This is a joint study with Dr. B. D. Perkins, USDA-ARS, Fort Lauderdale. Evidence suggests that water hyacinths in Florida infested with the above arthropods are invaded readily and colonized by a variety of facultative pathogens and wound parasites resulting in severe leaf necrosis, senescence and/or root damage. Dead plants were often seen among severely infected water hyacinths. Among fungi isolated from insect-infested plants, the most significant pathogens are *C. zonatum* and *Nigrospora sphaerica* (Sacc.) Mason. As mentioned earlier, the former is more virulent on water hyacinth than the latter. Spraying *C. zonatum* on insect-infested plants might increase the biological stress

and reduce the vigor of water hyacinths, leading to a gradual decline in populations. Laboratory tests are underway to prove this hypothesis. The effect of *C. zonatum* on the two arthropods, and the role of other facultative parasites in causing disease on insect-infested plants also are being evaluated. *Cephalosporium zonatum* is perhaps the most likely candidate for large-scale field tests. Current studies on its host range, evaluation of its effectiveness on water hyacinth under natural conditions, and the analysis of its role in the decline of insect-infested plants would be coordinated in justifying a large-scale field use of *C. zonatum* in Florida.

Search for newer pathogens and phytotoxic microbial products for use against submersed aquatic plants are being continued. Field testing, or applications of any of these newer organisms or their products for biocontrol of weeds, will be carefully reviewed by experimentation to assure safety.

HOST SPECIFICITY, SAFETY, SURVIVAL AND PERSISTENCE

Adequate studies on the host range of any potential biocontrol agent are required to insure that it will not seriously affect economically and ecologically important plants and animals. It is preferable to use a biocontrol agent with extreme host specificity. Rust pathogens are among the most highly host-specialized. But even rusts generally require alternate hosts to complete their life cycles. Though serious attempts to find newer rusts of aquatic weeds have not been made, such endeavors when undertaken might prove futile. It was pointed out earlier that rusts of water hyacinth and alligator weed have not been found since their early discoveries about 50 years ago. At present, choice of plant pathogens to control aquatic weeds is limited to facultative pathogens, which generally infect a number of hosts. Under these conditions, safety to other plant species can be assured by use of sufficiently narrow host-range pathogens similar to *C. zonatum*.

Overall, usefulness of plant pathogens in biocontrol is too significant to arbitrarily dismiss on the basis of unsubstantiated fears concerning safety to the environment. Since none of our target weeds are useful as fresh food for man or his animals, dangers of ingestion of plant pathogens via infected plants are limited. The potential dangers of plant pathogens to fish or other aquatic animals or to the food chain in water need to be studied.

In determinations of the ability of *R. solani* to survive in lake water, Freeman (11) found that sclerotia were viable for over 26 months. This is a much longer duration of aquatic survival of this organism than reported by other workers (25, 26, 36). Though the pathogen may be capable of surviving and infecting water hyacinth from submersed inoculum, its persistence in water is likely to be limited to a shorter length of time than in natural soils (23, 32) under comparable temperatures. Moisture certainly reduces the viability of sclerotia of this

pathogen (22, 23, 32) and possibly of soil fungi in general (31). Accordingly, irrigation reduces the viability of soil organisms like *Helminthosporium* and *Alternaria*, while aiding in the dispersal of aquatic fungi (31).

Plant pathogens are present in soil, air and water as resting structures. Of these, soil and water are normal habitats for survival of microorganisms. In a study, "Pollution of irrigation water by plant pathogenic organisms," J. R. Steadman of the University of Nebraska has found the natural occurrence of many potential plant pathogens in irrigation waters of North Platte Valley of Nebraska (Steadman, personal communication). Special attention will be given to the presence of *Sclerotinia sclerotiorum* (white mold of field beans), *Xanthomonas phaseoli* (bacterial blight of field beans) and plant pathogenic nematodes. Of concern to us is the effect artificial inoculations will have on natural populations of pathogens, in water and in soils surrounding bodies of water. What effects would the survival of a substantially higher number of pathogenic propagules have on the environment? If the organism produces a phytotoxin, would the higher levels of toxin have adverse effects on nonhost plants and animals? These and other related questions should be examined before large-scale uses of any microorganisms are initiated. More studies in this direction are needed. Our studies in Florida would include evaluation of survival, persistence, and safety of any organism used in biocontrol of aquatic weeds. Concerning use of purified phytotoxic microbial metabolites, persistence in the environment will perhaps be the least troublesome problem. Since microbial toxins are products of metabolism, they should be subject to biodegradation, unlike some man-made chemicals.

SUMMARY AND CONCLUSIONS

The term "microbial herbicide" has been coined by T. E. Freeman to denote any microbial agent used in control of a plant, and includes microorganisms as well as their products. The potential values of plant pathogenic microorganisms as bioherbicides against water weeds are many. First, most plant diseases have the ability to destroy any plant species but rarely do so (39). This could be an advantage in that, once a balance is established between populations of the host and the pathogen, the system might be self maintaining. Microbial populations can be mass produced commercially, although no aquatic plant pathogen is currently being manufactured on a commercial scale. The presence of technology to produce pathogens commercially is also a favorable point while considering biocontrol of weeds with plant pathogens. Generally, animals are non-susceptible to plant diseases and thus might be safe from aquatic weed pathogens used in biocontrol. Strict adherence to such safety procedures as use of fairly host-specific organisms and monitoring of their survival and persistence in the field would be necessary. Although we are looking forward to expediency in field trials, and possibly in large-scale tests, safety to

the environment and mankind is of prime concern.

We have at our disposal facilities of the University of Florida Agricultural Experiment Station and excellent cooperation of the following agencies: Florida Department of Agriculture and Consumer Services, U.S. Department of Agriculture, Florida Department of Natural Resources, U.S. Army Corps of Engineers, and Office of Water Research and Technology (formerly Office of Water Resources Research) of the Department of the Interior. The latter three organizations have also provided financial support for our program. Hopefully these studies will contribute toward establishment of procedures and standards for future researchers on biological control of aquatic weeds with plant pathogens.

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DISCUSSION

GEORGE ALLEN: The importance of exotic aquatic weeds was not fully recognized in the southeastern United States until a national environmental pollution control program was initiated in 1970. The severity of the problem continues to increase yearly, requiring increased funding to control various aquatic weed species.

In 1971, the Florida legislature appropriated funds to develop methods of aquatic weed control under the direction of the State Department of Natural Resources. In order to cope with immediate aquatic weed problems and to develop long-range plans for the state, a cooperative program including the U.S.D.A. and state universities was organized. Although the majority of aquatic weed pests are currently controlled by the use of herbicides, several approaches, including importation and introduction of various natural enemies, are being investigated to reduce future use of chemicals.

The U.S.D.A. has been actively engaged in research on biological control of aquatic weeds since 1959. Program emphasis until 1967 was on alligator weed, resulting in successful introduction of three insect species from Argentina. In 1967, the U.S.D.A. established a laboratory in Argentina to isolate and evaluate insect enemies of water hyacinth and later establish PL-480 projects in Europe and Asia to conduct similar research on *Myriophyllum spicatum* and *Hydrilla verticillata*. In the inter-agency cooperative program, the University of Florida was assigned the responsibility of conducting new foreign exploration programs.

Some of our most successful introduced and potential species are insects, therefore, we are very much concerned with their interaction with pathogens. The introduction of insects free of their predators and parasites is an important rule in biological control, however, their association with pathogens is often overlooked or not considered important. In the case of aquatic or semi-aquatic insect enemies of water weeds, pathogens are often the most important biotic factors involved.

Currently, water hyacinth, *Eichhornia crassipes*, is our number one aquatic weed pest in Florida. It has been proposed to be native to South America where it has an extensive range centered on northern Brazil and Venezuela.

Several insect species have been isolated and screened for importation at the U.S.D.A. laboratory in Argentina. The first introduction was the weevil, *Neochetina eichhorniae*. Limited releases were made in Florida in 1972, however, due to the possible simultaneous introduction of a microsporidan disease associated with the insect in Argentina, further releases have been delayed. Extensive surveys have been conducted in Argentina to determine the interrelationship of the pathogen, a *Nosema*, and *Neochetina eichhorniae* and *N. bruchi*, also scheduled for release in Florida. A selective breeding program was developed in Argentina to produce disease-free stock material which was examined in the United States before field release. Recently, we have encountered a

potentially more important parasitic nematode in both *Neochetina* spp. in Argentina. The nematode is a member of the order Tylenchida, many of which may cause a significant reduction in fecundity or adult mortality. Natural populations of both weevil species are susceptible to *Beauveria brongniartii* (= *B. tenella*) in Argentina. We have recently isolated this pathogen from field collected *N. eichhorniae* in Florida.

Another area of interest concerns the microsporidan diseases of *Acigona infusella* and *Epipagis albiguttalis*, two lepidopterous insects being considered by the U.S.D.A. for introduction on water hyacinth. Preliminary indications are that the pathogen associated with the larvae of both these species is *Nosema necatrix*, a major natural control agent of our native *Arzama densa*. The disease is so severe in both *Acigona* and *Epipagis* in Trinidad and Argentina that it has prevented laboratory rearing for basic studies. If a final diagnosis substantiates our findings, the decision to continue quarantine studies of these two insects should be reconsidered in view of the fact that both species inhabit the same niche as *Arzama densa*. It is also associated with the lily borer, *Bellura gortynoides*, a close relative of *A. densa* which attacks the yellow water lily, *Nuphar advena*, in Indiana. This species is also susceptible to a multiploid virion nuclear polyhedrosis virus.

The submerged plant *Hydrilla verticillata* was first observed in Florida in 1960 and is rapidly becoming our number one aquatic weed pest. We are currently involved in establishing the distribution and area of origin of hydrilla to enable us to develop foreign research projects in the search for natural enemies of the plant.

A U.S.D.A.-sponsored PL-480 project in Pakistan has yielded several insects associated with hydrilla. Three species, the dipterous leafminer *Hydrillia* sp., the weevil, *Bagous lutulosus*, and *Nymphula diminutalis*, show potential as biological control agents. Diseases have not been reported in any of these insects, however, based on our experience with hyacinth insects, we expect to encounter pathogen involvement.

Water lettuce, *Pistia stratiotes*, is a potential pest in many countries including the United States; therefore we are in the process of surveying for potential biotic factors. It is attacked in Florida by the Pyralid moth, *Samea multiplicalis*, by the weevil, *Neohydronomus pulchellus*, and a Noctuid moth in East Malaysia. *Samea multiplicalis* has been reported to be attacked by a nuclear polyhedrosis virus as well as by several parasites.

Although *Salvinia* spp., water fern, are not considered major worldwide or Florida aquatic weed pests, considerable research has been conducted to evaluate insect enemies as biotic agents as the result of the explosive growth of *S. molesta* on Kariba Lake in Africa. Three insects, the Acridid grasshopper *Paulinia acuminata*, the weevil *Cyrtobagous singularis*, and *S. multiplicalis* have been evaluated. *Metarrhizium anisopliae* is a significant pathogen of *P. acuminata* as well as *Cornops* sp., another grasshopper that attacks water hyacinth.

Aquatic plants provide both habitat and food for animal vectors of human diseases such as malaria, filaria, fascioliasis, and schistosomiasis (bilharziasis). *Anopheles*, *Culex* and *Mansonia* mosquitoes breed in the calm, still pockets of water created among stands of floating and emergent vegetation. In a study made in the Tennessee Valley Authority reservoirs, it was determined that *A. quadrimaculatus* breeding was related to the extent that plant organs intersect the water surface. *Pistia stratiotes* and *E. crassipes* are important species involved.

The intermediate hosts of schistosomiasis are species of the aquatic snails *Bulinus*, *Biompharia*, and *Oncomelania*, which live in the microhabitats provided by aquatic vegetation in which they find both shelter and food.

In Egypt, bilharzial snails are closely associated with several aquatic plant species including water hyacinth.

In summary, the control of aquatic weed pests is directly related to use of microorganisms to control aquatic insects. Insects have been and will be introduced in the future to control water weeds. Many of these insects are attacked by disease organisms, therefore, it would be wise to consider their susceptibility to any microorganisms proposed for aquatic insect control.

G. E. TEMPLETON: I want to begin with a compliment and a word of encouragement to Dr. Charudattan on his work with plant pathogens for control of water weeds, and Dr. Howard Ohr, who is also working in the area of biological weed control with plant pathogens. I commend both of these young men for their courage to enter this new and challenging research area and to set out on the arduous task of developing the scientific base of data with which to make a rational judgment on whether to introduce foreign pathogens for biological weed control purposes. As many of you know, plant pathologists are from 30 to 50 years behind entomologists in this area of introducing microorganisms for biological control.

Perhaps a reason is that in the history of plant pathology we have so many examples of introduced pathogens that have been devastating to man's crops and trees. I'm sure you're aware of the Dutch Elm disease, of Chestnut Blight, and Late Blight of Potato that precipitated the great Irish Famine. Every beginning plant pathology student knows these tales. I remember, too, how coffee rust completely devastated this crop in Ceylon and now threatens coffee crops in the Western Hemisphere because of its recent introduction into South America. There is also the story about how Charles Valentine Riley, an entomologist from Missouri, played a role in introducing downy mildew into France and nearly decimated the wine industry there while importing Phylloxera resistant root stocks from this country, so you can readily see why pathologists have been reluctant to undertake biological control in the classical sense.

I would like to offer another observation that I feel is very important in planning for future work in biological control. Progress can

be most rapid when the team approach is used with one specialist on the target organism and one on the biocontrol agent. This is how Dr. R. J. Smith, USDA-ARS weed scientist, and I have worked as we developed a biocontrol system for a leguminous weed in Arkansas rice fields. The team approach largely accounts for the success of this project.

Our objective is to demonstrate that biological control of weeds in cultivated crops is practical with fungal plant pathogens. Specifically we are seeking to demonstrate that endemic, rather than introduced, plant pathogens can be used for biocontrol of weeds in cultivated crops by employing them as bioherbicides, e.g., a mycoherbicide in this particular case. Our host-parasite combination is northern jointvetch (*Aeschynomene virginica* (L) B.S.P.) and the endemic fungal pathogen of jointvetch anthracnose, *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *aeschynomene*.

The weed problem that can develop in rice fields can reduce the rice yield but probably of equal economic importance is the dockage farmers take because of the trouble millers must go to so as to remove the black shiny seeds that are intolerable in packaged rice. The weed can be controlled with 2,4,5-T but there are several disadvantages to its use. As in this case the farmer did not want to spray his rice with 2,4,5-T for fear of it drifting to and damaging his neighbor's soybeans. Also, if 2,4,5-T applications are not properly timed the rice, too, can be damaged.

Careful examination of plants in a weed colony will reveal widespread and often uniform distribution of the anthracnose disease. It occurs naturally in practically every colony we have looked at, yet it is not particularly damaging to the weed. Thus it fits well the definition of an endemic disease. Interestingly, we have another related weed species, *A. indica*, quite widespread in our state but we have never been able to find this disease occurring on it. We have been able to infect *A. indica* in the field and greenhouse but the fungal isolates are not very virulent to it.

The typical anthracnose lesion of this disease has pustules (acervulae) of spores erupting from the tissue. These spores (conidia) are quite sticky, not readily disseminated by wind but are distributed in a passive way by insects of various types.

We isolated the fungus in pure culture on PDA or Lima bean agar and masses of spores are readily produced on the surface of the agar. It is a fungus that is very easy to work with in this respect. Photomicrographs show that the spores are relatively fragile. They are sufficiently stable for easy culture and harvest and spread in the field, yet surely not a type that would over-winter well in our climate.

In order to determine the host specificity of this pathogen, we have tested about 150 species and breeding lines of various crop and native plants in greenhouse and field tests. We've tried to emphasize soybeans, other leguminous plants and, of course, all the commercially important crops in our area such as cotton, rice, corn, etc.

We store isolates of the fungus in sterile soil, a standard procedure for storing fungi of various types. Transfers are made to modified Richard's solution, which is a sucrose-potassium nitrate medium supplemented with V-8 juice. It is my understanding that we'll have to substitute something for V-8 juice in this medium if we are to commercialize this fungus. It also requires relatively high aeration for good spore production.

In our field tests, after establishing a fairly good population of the legume weed, the treated plot has 100 percent control of the jointvetch 10 days after application of spores without damage to the rice or the grass weed.

The following table shows some representative data from our field tests. We have been testing it in the field since 1969 but we haven't found much difference in the effects of spore concentration (from 2 to 15 x 10^6 spores/ml) or plant height. In fact, we haven't found any situation in the field where it would not effectively control this weed. Last year we increased our test to a total of 80 acres using commercial equipment for application. It was effective in a variety of field situations including dried rice fields, flooded rice fields, young weeds, old weeds, etc., giving excellent control in every case.

Fungus on Northern Jointvetch, Field 1970

Plant height (cm)	% Control at indicated spores/ml x 10^6			
	2	3	6	Avg
10	100	86	86	91
25	86	86	72	81
58	86	72	57	72
Average	91	81	60	

Spray applied at 1300 L/ha.

We feel this fungus has commercial possibilities and it is our hope that with the help of the Upjohn Company we will be able to produce enough spores for testing on 500 acres this coming season, if we can satisfy Dr. Engler and the others of EPA about its safety to the environment and its toxicological safety. We are using around 15 ml of spore paste per acre as it is spun down from the culture medium and washed, versus 1500 ml of 2,4,5-T required to control the weed. Also, compared with levels of *Bacillus thuringiensis* used it is quite low. Fifteen milliliters of spores in 10 gallons of water applied to an acre containing a 6-inch flood without any interception by rice plants would amount to about 125 spores per ml. And since we are only adding to the native level of this fungus already present in this environment, I don't feel we are inducing a very significant perturbation of the environment.

Finally, I would like to say that every weed plant has from one to

one hundred or more diseases of various types. We feel that development of certain ones of these diseases as bioherbicides is a viable alternative to many chemical herbicides in cultivated crops.

JOHN BRIGGS: It seems that problems of formulation, application, registration, and identification are similar in plant pathology as well as insect pathology. I trust we will be able to maintain a community of plant and insect pathologists and identify this community with particular reference to those areas of safety, application, mass propagation, and application of pathogens of insects and weeds. From what Dr. Allen and the principal speaker said, we have in these integrated programs one of those magnificent opportunities in biology which has never been done satisfactorily in an educational institution--to bring together plant and animal sciences.

IGNOFFO: This was one of the ideas that we attempted to foster here. The problems are going to be common, and I don't think we have to restrict ourselves to potential biocontrol of weeds and those of insects. There are pest systems all over the world that we can address ourselves to, if we just broaden our horizons a little. I think many of these problems are very similar.

MARSHALL LAIRD: We're tending to label the beautiful water hyacinth with an unfortunate image, due to the fact that in considerable parts of the world *Eichhornia* has become a thundering nuisance. But in some areas, specifically Malaysia and Singapore, it's common practice for Chinese smallholders to maintain a pond alongside the house in which water hyacinth is grown and fed to the pigs. And I'm wondering whether, when we're considering the massive destruction of *Eichhornia crassipes*, we might have devoted little thought to replacement organisms which, in places like Southeast Asia for example, might be able to achieve the same job as pig feed that *Eichhornia crassipes* achieves now.

ALLEN: It's interesting that water hyacinth is utilized extensively as pig feed in Southeast Asia. But no good is accomplished by doing this, because water hyacinths are about 92-93% water and are poor fodder plants.

CHARUDATTAN: I made a point in my talk that none of these target aquatic weeds which we are attempting to control are used as fresh food for man or his animals. This is a generalization based on the fact that *Eichhornia crassipes*, *Hydrilla* and the others are not food plants. There are instances, I believe, in Louisiana, where cows are allowed to graze on alligator weed and water hyacinth. However, these are not usual pasture plants and have only limited value as food for animals.

J. E. ZAJIC: If you're going to remove an aquatic weed can't you expect something to replace it? What would be the replacement system? You're

dealing with an ecological problem, in which there is a delicate balance. The weed may not be what we want, but unless you have something to replace it, I can't see what the value is going to be to remove it. When we're growing crop plants, as in rice fields, I see a real value in weed control.

CHARUDATTAN: You are concerned about filling in the ecological niche, once you remove water hyacinth, or hydrilla. Probably we'll have a problem with one kind of weed or another during all our lives, and perhaps even our grandchildren's. We know, in our test systems, in small ponds and in aquarium tanks, once leafy hydrophytes, like hydrilla or water hyacinth, are removed, algae come in. Perhaps if we could accomplish something like aquatic weed management we could try to introduce some of our native plant species which always seem to be confined to their particular ecological niches.

JOHN PASCHKE: One of the classic examples of weed control using insects was with Klamath weed in California and other western states. Two species of beetles were introduced against the Klamath weed which led to a balance between the weed and the beetles. The Klamath weed is still there but large acreages of grazing land have been reclaimed. In Australia, cactus, *Opuntia* spp., is still present as a weed but in balance with its biocontrols, the insect and disease. The latter occur as a result of the attack of the lepidopterous insects that bore into the cactus pad. So, the biological control does not usually result in the total elimination of a target host.

ALLEN: I might make one comment here in reference to Dr. Zajic's question. All the plants we're dealing with in the aquatic environment of the southeastern United States are exotic species. Furthermore, we will never remove the environment of those plants that are here. With alligator weed, we do have some success, but some of this weed will always be there. At this reduced level it may be beneficial for fish. We need to realize that the reason we have these problems is that the reproducibility of hydrilla, *Pistia* and *Eichhornia* is tremendous. For instance, within one summer one plant of water hyacinth can be expected to vegetatively give rise to 5,000 offspring. These plants reproduce vegetatively and sexually but more so vegetatively. We're always going to have these plants in Florida. All we're trying to do is to coordinate our efforts in reducing the populations of these plants to where we can live with them.

E. I. HAZARD: I have to address this to Dr. Allen. Two questions: First, I wonder if you'd comment on the role that the manatee is playing, if any, in the biological control of water hyacinth. And second, I recall a study which pointed out that water hyacinth was beneficial, being capable of trapping a lot of the excess nutrients which would have led to eutrophication, algal bloom, and depletion of oxygen. I just

wonder if you'd comment on the possible beneficial effects, if any, of the water hyacinth.

ALLEN: The manatee is native to Florida and one of the first control agents we thought of. But its requirements for reproduction are so fine--it needs certain water temperature and so forth--that it will never be feasible.

In reference to the beneficial part, hydrilla, water hyacinth and most of these aquatic plants are able to take up and store tremendous amounts of nutrients, up to 9-10% times more than they need. If we could remove the plants easily, we could put them in enriched waters, remove the nutrients, and clear the plants once the water is clean. Water hyacinths have been found to be very useful in this respect. We're going to use water hyacinths in a sewage plant where they would be very beneficial in removing nutrients from the sewage.

D. W. ANTHONY: I just want to make one comment on the manatee. This animal was tried in the Chagres River in Panama, as I understand, for about 3 or 4 years before it was finally given up. It just didn't work at all.

BRIGGS: There were a couple of comments with respect to metabolic products of mircoorganisms, their possible use as insecticidal or herbicidal materials, and about their possible degradation. I wonder, Dr. Singer, if you would tell us if microbial metabolites are easily degraded?

SINGER: We don't know much about these toxins; there haven't been many studies. The only way to establish the toxicity of a substance is to biologically assay it. With antibiotics, like penicillin, there are cases in which some organisms split the penicillin part. This is probably true of most antibiotics and microbial products, most of which probably are biodegradable. As with microorganisms used as control agents, we have to consider them in relation to total population. Presumably we will be putting them into competition with natural populations. Hopefully, they will be viable enough to maintain this feedback situation--as the host increases, they will attack it and so forth.

IGNOFFO: There's a very excellent example of exotoxin in which the removal of the phosphate radical leads to its complete deactivation. There are several other examples of such degradation of compounds in nature.

I want to close with a few remarks. There's a series of publications that might be helpful in this area. One is the WHO Report, No. 531, available through Dr. Arata, on the use of viruses for control of insect pests and disease vectors. It goes into some of the problems, safety requirements, registration, etc., for development of viruses.

There's another WHO publication by Ray Smith, called "Considerations on the Safety of Certain Biological Agents for Arthropod Control." More recently, there's been a publication in the Proceedings of the New York Academy of Science which covers this whole area. Dr. Singer referred to a book, edited by Burgess and Hussey, on microbial insect control. These may be more helpful to those people interested in this area to see how it might relate, and solve some of the problems they may be exposed to.

The area of biological control is a very exciting field with great potential. We need to find other ways, but not to the total exclusion of chemical pesticides. We should be able to prudently use these chemicals in integrated systems to control aquatic pests. The major concern is that microbial controls will mutate or infect non-target organisms, or will eventually get into an ecosystem and affect other animals.

II

ENVIRONMENTAL CONSIDERATIONS ON MICROBIAL DEGRADATION OF OIL

BACTERIAL GROWTH AND DISPERSION OF CRUDE OIL IN AN OIL TANKER DURING ITS BALLAST VOYAGE

E. Rosenberg, E. Englander, A. Horowitz and D. Gutnick*

ABSTRACT

A compartment of an oil tanker, containing 107 m^3 oily ballast water, was supplemented with 7.6 mM urea, 0.57 mM K_2HPO_4 and 3 m^3 air per min. After 4 days, oil dispersion became evident and bacterial concentration reached over 10^7 cells/ml. When the treated water was discharged, no sign of oil was detected in the ship's wake. A non-aerated control compartment, containing 121 m^3 oily ballast water, yielded 10^5 cells/ml and no oil dispersion. In order to understand the mechanism of bacterial-induced cleaning of oil tanks, the emulsifying agent produced by RAG-1, an *Arthrobacter* sp., has been partially purified and characterized. The purified agent, E_{RAG}, forms stable emulsions with over 200 times its weight of crude oil, gas-oil or hexadecane. E_{RAG} is resistant to 100 C, excess KIO_4 and pronase; the half-life of E_{RAG} was 5 min in 1 N HCl at 100 C and 2 hr in 1 N NaOH at 100 C. RAG-1 induced oil emulsions were toxic to developing sea urchin embryos. However, emulsions formed with E_{RAG} showed only slight toxicity which could be removed with small dilutions into sea water. An oil-degrading bacterium, referred to as UP-2, has been isolated by enrichment culture with RAG-1 depleted oil as the nutrient. UP-2 has a broader substrate specificity than strain RAG-1.

INTRODUCTION

There have been a large number of reports (e.g., 1, 3-5, 8, 10, 11-13) describing the properties of pure and mixed bacterial cultures capable of dispersing and degrading crude oil in supplemented sea water. In each instance, the ability of the bacteria to significantly degrade oil was entirely dependent on an exogenous source of nitrogen and phosphorus. This requirement for supplemental nitrogen and phosphorus compounds most likely accounts for the very slow breakdown of oil in the open sea, and suggested to us that the utilization of microorganisms in the treatment of oil pollution can be more easily accomplished in situations in which the polluting oil is confined. One such environment is in the cargo compartment of an oil tanker.

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After delivering its cargo of crude oil, a petroleum tanker must take aboard large quantities of ballast water before putting to sea. This ballast water mixes with residual oil in the cargo compartment (between 0.1 and 1.0%). Disposal of this heavily contaminated oily ballast water is a major contributor to chronic sea pollution. In addition, cargo compartments of oil tankers must be cleaned periodically in order to prevent clogging and sludge accumulation which would otherwise reduce the ship's cargo capacity--and inhibit discharge of crude oil. Presently, cargo compartments of tankers are usually cleaned with high pressure jets of sea water; the combined ballast and wash waters are then either a) discharged at sea, b) transferred to separator tanks on shore where harbor facilities permit, or c) transferred to small settling tanks on deck ("slop" tanks) in which the water at the bottom is removed and additional oily ballast water is loaded on top (load-on-top technique). Prior to dry dock, additional scrubbing by hand, or removal of fine particles with the aid of vacuum cleaners is required.

As part of a general program on degradation and dispersion of crude oil by bacteria, we decided to explore the possibility of utilizing bacteria for cleaning cargo compartments of oil tankers. We report here a) the growth of bacteria on supplemented oily ballast water within a cargo compartment during its ballast voyage, b) certain chemical and biological properties of a bacterial-produced dispersing agent, and c) the isolation of oil-degrading bacteria by sequential enrichment culture procedures.

THE SHIP EXPERIMENT

Prior to the experimental voyage, several experiments were conducted (mostly on a 10-liter scale) under a variety of conditions that might be encountered during the voyage. The following general conclusions were reached: a) Using 0.3% crude oil in aerated sea water, maximum oil dispersion and bacterial growth yields were achieved with 7.6 mM urea and 0.57 mM K_2HPO_4. b) In the early experiments, ammonium sulfate was used as the nitrogen source, resulting in a final pH of 5-6. This acidic pH is inhibitory for further development of bacteria and could be detrimental to certain metal components in ship tanks. By using urea instead of ammonium sulfate, we were able to maintain the pH between 7.6 and 8.1, independent of the temperature and oil concentrations chosen for growth. c) Similar kinetics of oil dispersion and bacterial growth occur over a rather wide range of temperatures, 20-37 C.

In order to perform the ship experiment, we were provided with the use of the two "slop" tanks on a 120,000-ton oil tanker. During the ballast voyage prior to the experimental trip, a device for providing air was installed in the starboard "slop" tank (Tank A). Both tanks were subsequently filled with Agha Jari light crude oil (2). The aeration system consisted of segments of polyethylene hose, 32 mm in diameter, connected by polypropylene fittings such that the hose covered the bottom surface of Tank A. Fifty holes, 2 mm in diameter, were drilled throughout the hose so as to provide equal distribution of air. This device

was connected to a valve on deck by a long hose of the same diameter (32 mm).

We boarded the oil tanker which was in the process of discharging its cargo in Eilat. The following day, after discharge was completed and the ship had left port, ballast water was added to each of the two tanks, 2.48 meters in Tank A, and 2.70 meters in the port "slop" tank (Tank B). The total volume of liquid in Tank A was 107,000 liters and that in Tank B, 121,000 liters. Twenty kilograms of urea and 1 kg K_2HPO_4 were dissolved in sea water and added to each tank. Air was then introduced into Tank A at the rate of 3,000 liters/min. Duplicate samples were removed from each tank at time "zero." Subsequently duplicate samples were taken 3 times daily from Tank A and once daily from Tank B for the following 157 hr. In addition, duplicate samples were withdrawn from the full oily ballast tank No. 4 at 137 hr. Measurements of oil dispersion and viable cell number (10) as well as microscopic observations of the samples were made in the Deck Office which served as a laboratory during the voyage.

At the beginning of the experiment, a thick layer of oil could be seen floating on the surface of the water in the tanks. The oil layer in Tank B remained in this form throughout the course of the experiment. Starting at 93 hr, the appearance of the oil surface in Tank A began to change. The oil began to coagulate, and streaks could be seen on the surface. The oil in the samples taken from Tank A started to change dramatically at about 100 hr. In these samples the oil appeared very "mushy" (probably a water-in-oil emulsion) with the consistency of a pudding. With increased time, more and more of the oil was found to disperse into the water phase (Fig. 1). In sharp contrast to what was observed during the early sampling periods, when the oil adhered very tightly to the walls of the sample bottle, by the end of the experiment a slight shaking of the container resulted in a complete disappearance of oil from the walls of the flask. No such dispersion and cleaning was observed in any of the samples from Tank B or Tank No. 4.

Figure 2 summarizes data on viable cell number during the course of the experiment. These are minimum values since only those bacteria which can form colonies on nutrient agar are measured. The initial concentrations of bacteria in Tanks A and B, as well as in the unsupplemented control Tank No. 4, were more than 10 times higher than in sea water sampled alongside the ship just prior to taking on ballast water. The probable source of these bacteria was the residual oil in the tanks. After a lag period of about 22 hr, the bacteria concentrations increased during the subsequent 72 hr, about tenfold in Tank B and 1000-fold in Tank A, yielding about 1×10^5 and 2×10^7 viable cells/ml, respectively. Total cell number, as determined by use of a Petroff-Hauser counting chamber, on formaldehyde fixed samples gave values 5-10 higher. The protein concentration in Tank A at 156 hr was 95 mg/liter as determined by the Lowry method (6) on cells harvested at 15,000 x g for 30 min. This value is also minimal, because some protein was detected in the supernatant fluid following the centrifugation.

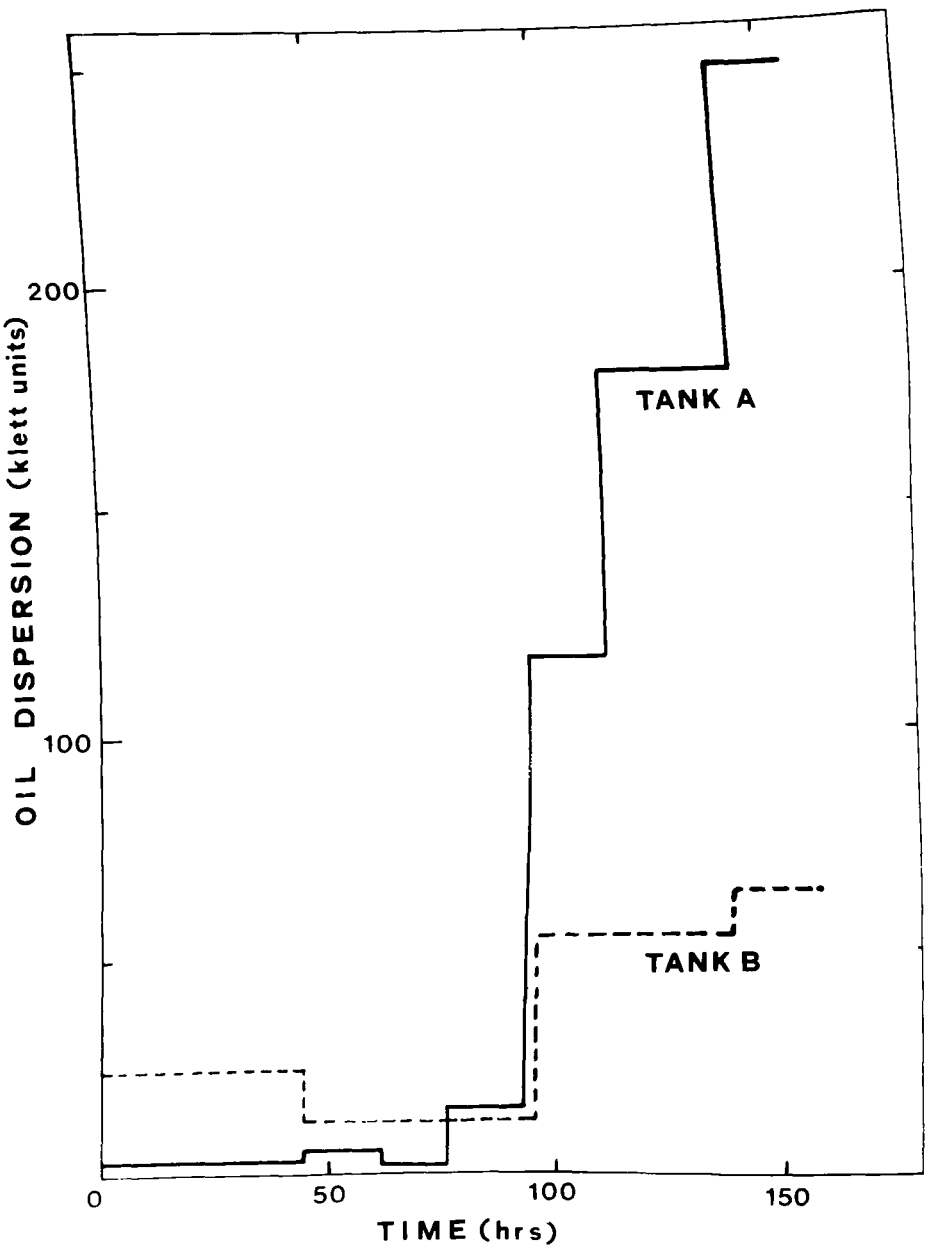

FIGURE 1. Oil dispersion inside the cargo compartment of an oil tanker during its ballast voyage. The oil ballast water in Tanks A (107,000 liters) and B (121,000 liters) were each supplemented with 20 kg urea and 1 kg potassium phosphate. Tank A was aerated at the rate of 3,000 liters/min. Tank B served as a non-aerated control. Oil dispersion was estimated from the turbidity of the oil-water suspension after allowing 2 min for the non-dispersed oil to separate (see ref. 10 for details of this method). Time zero corresponds to the time of addition of ballast water and supplements to the residual oil in the tanks.

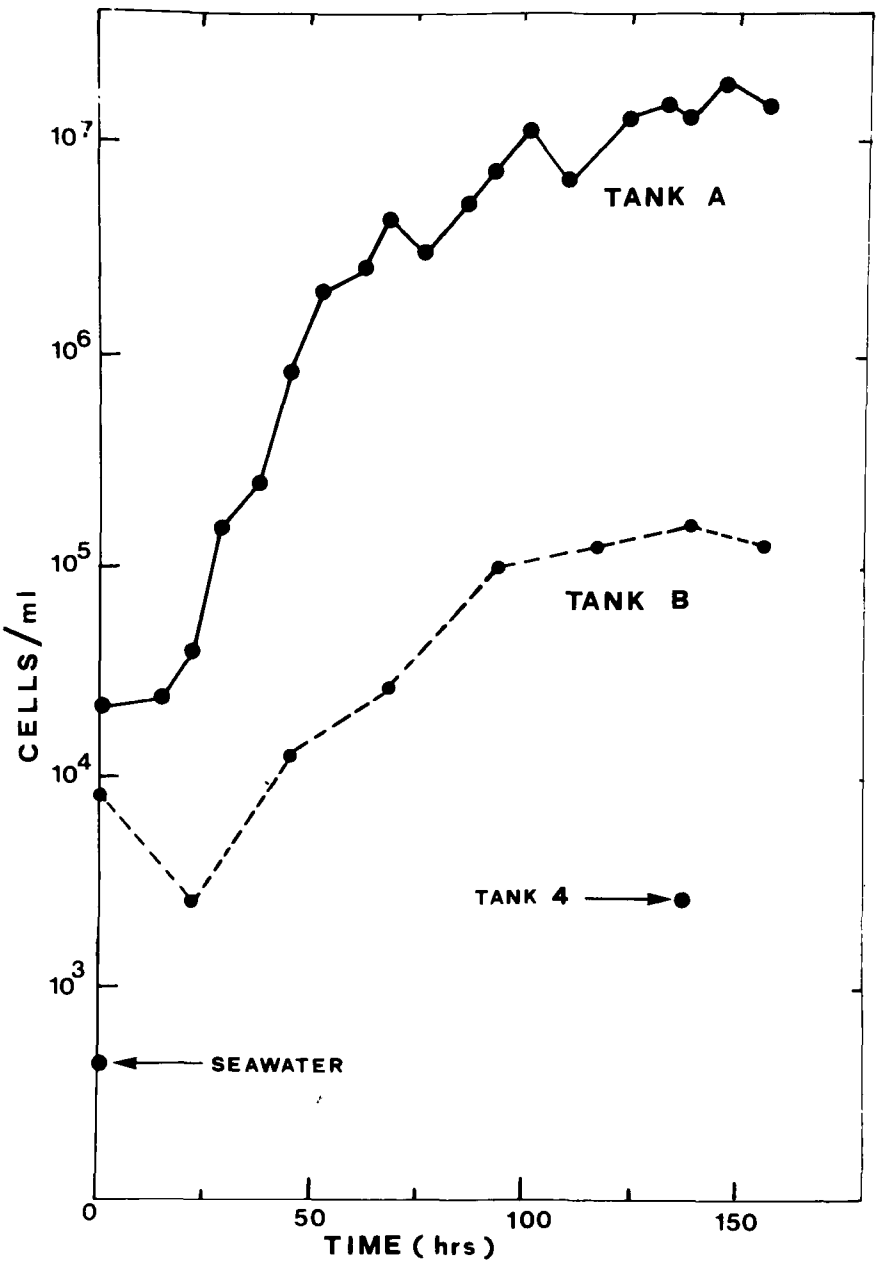

FIGURE 2. Growth of bacteria inside the cargo compartment of an oil tanker during its ballast voyage. The conditions are those described in Figure 1. The ordinate indicates the viable cell number as determined by plating an appropriate dilution onto Nutrient Agar. Tank 4, a full normal ballast compartment, served as an unsupplemented control. Sea water sampled from alongside the ship just prior to taking on ballast water is shown for comparison.

Throughout the experiment, the temperature remained between 23 and 25 C and the pH between 7 and 8, in both the experimental and control tanks.

Tank A was emptied after 156 hr, and during that procedure no material could be observed which darkened the appearance of the white foam in the wake of the ship. When Tank B was subsequently emptied, we observed the expulsion of a thick black material followed by a yellow oil slick which appeared in the wake. After Tank A was vented, we entered the tank with the Chief Mate. The bottom and lower 2.5 meters of the walls of the tank were found to be completely free of the thick layer of sludge which had accumulated on the ladder, platforms, and upper portion of the walls of the tank. There were almost no residual oil stains along the bottom surface.

PROPERTIES OF DISPERSING AGENT PRODUCED BY RAG-1

In order to better understand the mechanism of bacterial cleaning (oil dispersion) of oil tankers, we have investigated the production of extracellular dispersing agent(s) produced by the *Arthrobacter* sp. strain RAG-1 (1) growing on hexadecane as the sole source of carbon and energy. The evidence indicates that bacterial growth and production of dispersants are not necessarily correlated. Maximum oil dispersion and production of the extracellular dispersing agent occur 1-2 days after the bacterium reaches stationary phase. Furthermore, RAG-1 grows well, but does not produce any measurable dispersing agent, on several nutrients, such as glucose, succinate, hexadecanol and hexadecanoic acid. Optimum production of dispersing agent occurs when the cells are grown on straight chain paraffins from pentadecane to nonadecane. We do not yet know if the paraffins are necessary substrates for biosynthesis, or play a regulatory function leading to the production of dispersants.

The oil-dispersing agent has been partially purified from stationary phase cultures of RAG-1 growing on 0.1% hexadecane, 0.058 mM K_2HPO_4 and 7.5 mM urea in sea water. After removal of cells by centrifugation at 10,000 x g for 15 min, the clear supernatant fluid was dialized for 18 hr against distilled water, concentrated *in vacuo* and then precipitated with 4 vol acetone containing 1% LiCl. The precipitate was dissolved in 0.01 M phosphate buffer, pH 7.2, and passed through a Sephadex G-200 column. The void volume which contained all of the emulsifying activity was dialyzed extensively against distilled water and lyophilized to yield the partially purified dispersing agent E_{RAG}. An amount of 250 mg dry white powder per liter culture fluid was obtained.

Purified E_{RAG} rapidly forms stable emulsions with over 200 times its weight of crude oil, gas-oil or hexadecane. Although E_{RAG} is eluted in the void volume following chromatography on Sephadex G-200, it can be dissociated into active dialysable subunits. In dilute aqueous medium, E_{RAG} appears microscopically as micelles. There is no measurable loss in activity of E_{RAG} by the following treatments: (1) 100 C, 1 hr; (2) 1 mg/ml Pronase, 37 C, 18 hr; (3) excess KIO_4. The half-life of

E_{RAG} was 5 min in 1 N HCl at 100 C and 2 hr in 1 N NaOH at 100 C. A report on the chemical and spectral analyses must await a demonstration that the material is homogeneous.

The toxicity of crude oil and dispersions of crude oil to developing sea urchin embryos are shown in Table 1. The developing sea urchin embryo system was utilized because of the possibility of subsequently examining the biochemical basis of the toxicity. The data clearly indicate that bacterial emulsions of crude oil are over 100 times more toxic than the crude oil itself. The toxicity of the bacterial-induced emulsions can be decreased over 20-fold by dialysis against sea water. Table 2 indicates that the purified E_{RAG} is not toxic whereas emulsions formed by E_{RAG} are slightly toxic. When the E_{RAG}-oil emulsion was diluted 1:5 into sea water, no toxicity was detectable. It should be pointed out that these measurements may differ from previous determinations of toxicity of marine organisms (7) or inhibition of chemotaxis (9).

SEQUENTIAL ENRICHMENTS FOR OIL-DEGRADING BACTERIA

Like many oil-degrading bacteria, RAG-1 accomplished only a partial breakdown of crude oil. Under conditions of limiting oil concentration, approximately 50% of the oil is converted by RAG-1 into a form which is no longer extractable by organic solvents (10). In order to isolate additional oil-degrading bacteria, the residual oil (RAG-1 depleted) was recovered and added to sea water supplemented with urea and K_2HPO_4. This medium was then utilized for a second enrichment culture procedure. The dominant microorganism, referred to as UP-2, appearing in the secondary enrichment culture was isolated in pure culture for further study.

Table 3 shows the growth of RAG-1 and UP-2 on different carbon sources. Both RAG-1 and UP-2 grew well on crude oil or straight chain paraffins derived from crude oil. Neither strain was capable of growth on the asphaltene fraction of oil. Whereas RAG-1 grew poorly on branch chain paraffins or oil that had been depleted by prior growth of RAG-1 or UP-2, the strain UP-2 grew relatively well on branch chain paraffins and RAG-1 depleted oil, but poorly on UP-2 depleted oil. These data indicate that UP-2 has a broader range of substrate specificity than RAG-1. These preliminary experiments indicate the potential of utilizing "bacteria-depleted oil" as substrates for the isolation of new strains of oil-degrading bacteria. It should be emphasized that UP-2 never appeared in our initial enrichment cultures on crude oil.

TABLE 1. Toxicity of Crude Oil and Bacterial Dispersions of Crude Oil to Developing Sea Urchin Embryos

Additions to the suspension of embryos	Microscopic observations at the following stages					Conclusions
	1 hr	3 hr	24 hr	48 hr	72 hr	
1. None	2 cells	8 cells	gastrula	prism	late pluteus	Normal development
2. Crude oil 1%	2 cells	8 cells	gastrula	prism	few pluteus	Slight toxicity
3. Bacterial emulsion[a] of 1.0% oil, diluted: 1:4	1 cell	1-2 cells	rupture of embryo			Very toxic
1:40	1-2 cells	2-8 cells	never reaches gastrula			Very toxic
1:100	1-2 cells	2-8 cells	80% gastrula	10% prism	no pluteus	Toxic
1:200	2 cells	8 cells	gastrula	prism	late pluteus	Non-toxic
4. Dialyzed[b] emulsion diluted: 1:4	1 cell	4 cells	motile embryo but never reaches gastrula			Toxic-developmental arrest
1:6	2 cells	8 cells	gastrula	prism	no pluteus	Toxic
1:10	2 cells	8 cells	gastrula	prism	late pluteus	Non-toxic

[a]Four-day culture of RAG-1 grown on 1.0% crude oil in supplemented sea water was diluted into suspension of embryos as indicated.

[b]RAG-1 induced emulsions were dialyzed extensively against sea water prior to dilution.

TABLE 2. Toxicity of Bacterial Emulsifier (and the Emulsions) to Developing Sea Urchin Embryos

Additions to the suspension of embryos	Microscopic observations at the following stages					Conclusions
	1 hr	3 hr	24 hr	48 hr	72 hr	
1. None	2 cells	8 cells	gastrula	prism	late pluteus	Normal development
2. E_{RAG}[a] 0.25 mg/ml	2 cells	8 cells	gastrula	prism	late pluteus	Non-toxic
3. Emulsion formed by E_{RAG}	1 cell	2-4 cells	motile but deformed embryos			Toxic
1:3	2 cells	8 cells	gastrula	never reaches prism		Toxic-developmental arrest
1:5	2 cells	8 cells	gastrula	prism	pluteus	Non-toxic

[a] E_{RAG} was purified as described in the text.

TABLE 3. Growth of RAG-1 and UP-2 as a Function of Oil Fraction

Nutrient (0.1%)[a]	RAG-1/ml[b]	UP-2/ml[b]
1. Crude oil	6×10^8	2×10^8
2. RAG-1 depleted oil[c]	2×10^6	7×10^7
3. UP-2 depleted oil[c]	7×10^5	2×10^7
4. Straight chain paraffins	3×10^8	1×10^9
5. Branch paraffins	6×10^6	3×10^8
6. Asphaltenes	$<10^6$	$<10^6$

[a] In addition to the nutrient, the sterile sea water contained 0.058 mM K_2HPO_4 and 7.6 mM urea.

[b] Total cell number was determined with a Petroff-Hauser counting chamber.

[c] Depleted oil was prepared by growing the bacterium under conditions in which the crude oil was growth limiting. The residual oil was then extracted and concentrated.

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SELECTIVE ENRICHMENT PROCESSES IN RESOLVING HYDROCARBON POLLUTION PROBLEMS

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ABSTRACT

The existence of hydrocarbon-oriented continuous enrichment systems in nature is not unusual. This is substantiated by the additional one or two orders of magnitude of hydrocarbon-oxidizing microorganisms present per unit volume in chronically oil-polluted waters. The varying complexity of crude oils, as well as the presence of incomplete and co-oxidative metabolic intermediates, promotes the ubiquity of mixed cultures with diverse enzymatic capabilities. An enrichment approach requires evaluation of hydrocarbons, toxic hydrocarbons and high molecular weight hydrocarbons in terms of their potential as energy for microbes and how they impose contraints during biodegradation. Specific examples are given to demonstrate the ability of the selective enrichment process to resolve "organic pollution problems."

SELECTIVE ENRICHMENT

Recently a considerable amount of attention and research has focused on the microbial degradation of oil pollutants in terms of artificially seeding oil spills. In this regard, many studies have been conducted to determine the factors affecting biodegradation of hydrocarbons. The factors, including oxygen concentration, nutrients, temperature, turbulence, etc., were evaluated primarily in terms of their effect on rates of mineralization and decomposition, components of the hydrocarbon or oil attacked, and the extent to which these constituents were degraded. Many of the factors affecting hydrocarbon biodegradation have been considered elsewhere (1, 2, 4, 6, 25, 29).

In order to fully evaluate artificial seeding techniques, a number of other, perhaps more basic, considerations must be examined. What types of microorganisms should be used, which ones will predominate because of selective enrichment, what other artificial materials, i.e., nutrients, emulsifiers, etc., must be added to spills and, most important, what effect will all of this have on the naturally-occurring flora and fauna?

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It is generally agreed (4, 7, 21, 26, 36) that one genus or even one class of microorganisms cannot degrade all the components of crude oil completely. This is due to the complex composition of the crude oil, as well as the numerous physical and chemical constraints, i.e., temperature, toxic intermediates, salinity requirements, etc., that render a single type of microbe ineffective. The seeding of oil spills can be viewed as an artificial implantation of a widely mixed microbial population under probably non-ideal conditions on a highly variable system. Thus, it is likely that continuous selective enrichment condition will be important in determining the outcome. The more highly toxic the waste, the greater the need for an enrichment process, since this permits selection of mutants with a greater tolerance to the toxic compound and an increased ability to degrade the toxic component.

The concept of natural selection in the microbial world was formulated in the classic paper by C. B. van Neil (32). This somewhat Darwinian approach suggested that it is the environmental conditions that determine which biological forms can best compete in the struggle for survival. Single cell organisms are especially sensitive to environmental conditions because changes in temperature or composition of the microenvironment can cause related conditions within the microbe to change rapidly and often significantly. For example, it has been calculated (12) that a spherical particle, one micron in diameter, will reach the temperature of its environment within milliseconds even with the assumption of unrealistically low thermal conductivities. The large surface area to volume ratios and minute diffusional path lengths typical of the microbial world result in high metabolic rates. In addition, adaptation and natural mutation rates are generally high, and generation times are typically minutes or hours as compared to days or weeks for more complex life forms.

Microorganisms in pure or in mixed cultures are dependent upon such factors as composition and concentration of substrates, actual and induction enrichments, temperatures, availability of nutrients, etc. With mixed culture systems, however, factors such as the production of compounds toxic or inhibitory in very low concentrations to potential microbial competitors, or differences in growth rates, play important roles in determining generic composition and sequence of predominating organisms. Furthermore, in mixed populations gradual modification of the environment brought about by proliferation of one or more organisms inevitably makes the altered environment more favorable for development of other microbes. Selective enrichment considerations, then, are important in evaluating the effect of artificially-seeded mixed populations on oil spills in terms of types and degree of component decomposition, effect of degradation-enhancing materials, microbial species interactions and, ultimately, possible impact on the environment.

COMPOSITION OF OIL

One of the most important factors affecting generic composition of hydrocarbonoclastic microorganisms in oil spills, as well as the possible extent of biodegradation, is the composition of the crude oil. In a recent study of the bacterial seeding of oil slicks (21) it was suggested that the type and quantity of crude oil used, rather than nutrient concentration or inoculum density, determined the effectiveness of artificial seeding. Fractions of crude oil are often categorized as either *n*-saturated, aromatic, asphaltene or NSO (nitrogen-sulfur-oxygen); further classification to the percent of individual components in each fraction is sometimes determined as well.

It is generally agreed that the *n*-saturate fraction of crude oil is most readily attacked by hydrocarbonoclastic microbes (3, 10, 13, 18, 31, 35). Generally, the monoic acid pathway (alkane to primary alcohol to aldehyde to carboxylic acid) is the primary mechanism of alkane oxidation; dioic acid formation by di-terminal oxidation is thought, at best, to be only a minor pathway (31). It has been suggested (23) that degradation of individual *n*-alkanes does not occur at a uniform rate and that a polyauxie phenomenon may be active. This condition, however, is less likely to occur when mixed populations of microbes are used (20).

Although it has been shown that some relatively long-chain alkyl-substituted cyclic hydrocarbons, such as *n*-nonylbenzene and *n*-dodecylbenzene, can support microbial growth and form cyclic acids (11), the large proportion of alkyl-substituted cyclic hydrocarbons are only partially oxidized (co-oxidized) in the presence of *n*-alkanes (11, 16, 24, 31). Consequently, phenyl alkanes tend to be incompletely oxidized and may accumulate as phenyl-substituted fatty acids (11, 31). Microbial attack invariably is concentrated on the alkyl chain rather than on the phenyl or cycloalkyl ring (31); ω-oxidation of the alkyl substituent is followed by β-oxidation (11). Further degradation of cyclic acids with even numbers of carbon atoms in the fatty acid chain (phenylacetic, cyclohexaneacetic, cyclohexanebutyric) has been shown to be difficult, whereas cyclic acids with odd numbers of carbons in the fat acid portion (benzoic, phenylpropionic, cyclohexanepropionic) are readily degraded further (11).

The microbial degradation of aromatics has been reported in several summary papers (16, 31), yet detailed research into the exact mechanisms has been severely limited by the extremely labile nature of the enzymes that catalyze the initial incorporation of oxygen into aromatic hydrocarbons (16). Most available evidence indicates that catechol or an alkylated form of catechol is an intermediate of aromatic hydrocarbon biodegradation. For example, catechol has been shown to be a metabolic intermediate in the biodegradation of benzene and toluene; following the formation of catechol, the aromatic ring is split to produce a dicarboxylic acid. Naphthalene, anthracene and phenanthrene degradation proceed to catechol via salicylic acid (16, 31).

Considerable work has been done to examine the biodegradability and microbial metabolic pathways of hydrocarbon fractions, or more commonly, the degradation of specific components of hydrocarbon fractions by hydrocarbonoclastic isolates. This has led to generalizations, i.e., if a microbe is capable of utilizing one or more aromatic hydrocarbons, it will, in all likelihood, be able to utilize aliphatic hydrocarbons (10, 29); and, a microbe grown on a particular hydrocarbon will, in general, attack related but structurally more complicated hydrocarbons immediately, but without complete oxidation (31). However, the extent and relative rates of utilization by mixed populations of microbes has not been fully investigated to date. Such research hopefully would answer some of the questions implicit in the many investigations of hydrocarbon biodegradation by pure cultures and/or with pure substrates: What effect would the toxic or non-biodegradable hydrocarbons have on utilization of other fractions? How would accumulation of certain intermediates (cyclic acids) affect microbial attack of other hydrocarbons? To what extent can the polyauxie condition of paraffins be applied to the remaining fractions of the crude oil? Would microbial species interactions influence oil biodegradation? Which microbial species would predominate, and for what length of time?

A number of studies of biodegradation of crude oils by mixed populations of microorganisms have been conducted recently (2, 3, 17, 18, 33). Westlake and his co-workers (17, 33) determined that the composition of the crude oil substrate, as well as the composition of the enrichment oil, determined not only the generic composition of the microbial population but also the growth characteristics (see Table 1). Crude oils were classified from a biodegradability standpoint as being of high or low quality depending on the compositional relationship between saturates and aromatics. The studies determined that biodegradation depended not only on the metabolic capabilities of the microbes present and composition and amount of the paraffinic fraction, but also on the chemical composition of the asphaltenes and NSO fractions. Normal or high levels of *n*-saturates did not necessarily assure biodegradability. Kator (18) found that the rate of total *n*-paraffin utilization was proportional to the amount of this fraction present in the crude. It was found (17, 33) that microbial populations enriched on a poor quality oil readily attacked a high quality oil but that microorganisms enriched on high quality crude oil were invariably incapable of effectively metabolizing a low quality oil. Polar, non-hydrocarbon NSO's and asphaltenes apparently are produced as a result of microbial action on crude oils, resulting in a low-wax, lower API gravity crude oil (3, 33). Atlas and Barth (2) demonstrated the probable existence of a volatile toxic fraction in crude oils that had to be removed before effective biodegradation could occur. The reduction of a biodegradation lag period for "preweathered" crude oil samples versus fresh crude oil was demonstrated.

The preceding investigations indicate the wealth of relevant data obtainable from studies of the mixed population biodegradation of crude oil; further research in this area is certainly desirable.

TABLE 1. Change in Generic Composition[a] of Microbial Populations at 30 C as Affected by Crude Oil Composition[b]

	Percent of Population			
	Enrichment Oil	Test Oil		
	Lost Horse Hill	Norman Wells	Prudhoe Bay	Atkinson Point
Achromobacter sp.	67	56	56	35
Acinetobacter sp.	16	20	16	38
Flavobacterium sp. and *Cytophaga*	2	5	7	10
Pseudomonas	15	19	21	17
	Norman Wells	Prudhoe Bay	Lost Horse Hill	Atkinson Point
Acinetobacter sp.	28	20	16	22
Arthrobacter sp.	8	10	4	9
Xanthomonas sp.	16	26	26	3
Unidentified Gram negative rods[c]	16	11	11	19
Unidentified Gram negative rods[c]	9	17	8	31
Unidentified Gram negative rods[c]	24	15	34	15
	Prudhoe Bay	Norman Wells	Lost Horse Hill	Atkinson Point
Achromobacter sp.	18	20	15	17
Alcaligenes sp.	8	5	7	10
Arthrobacter sp.	46	45	44	12
Pseudomonas sp.	21	23	20	27
Unidentified Gram negative rods[c]	2	1	3	3
Unidentified Gram negative rods[c]	5	6	11	31
	Atkinson Point	Norman Wells	Prudhoe Bay	Lost Horse Hill
Achromobacter sp.	34	27	44	28
Alcaligenes sp.	27	24	20	29
Xanthomonas sp.	39	49	36	43

[a]After 4 days growth of the fourth transfer at 30 C.

[b]Adapted from Westlake et al., 1974 (33).

[c]Differing at least in one character from the designated genera.

TEMPERATURE EFFECTS

With the increased interest in tapping the large oil reserves in northern Canada and Alaska, the ecological impact of a possible oil spill in Arctic waters has received widespread attention and controversy. Cold ocean waters are not, however, unique to the Arctic areas since greater than 90%, by volume, of the world's seas are less than 5 C (37). It has been suggested (14) that the majority of marine microorganisms are probably psychrophiles, which has, in all likelihood, prompted several of the studies of hydrocarbon degradation at low temperatures (2, 10, 17, 26, 29, 33, 37, 38).

In studies by Westlake and his co-workers (17, 33) it was found that the temperature used during enrichment of hydrocarbonoclastic microbes had a significant effect on the generic composition obtained. Enrichments performed on the same oil, but at 4 C and 30 C, yielded substantially dissimilar microbial distributions (see Table 2). Cell yields and growth rates at 4 C were only slightly less than those at 30 C. This is perhaps in contrast with studies by Atlas and Bartha (2), Cundell and Traxler (10) and ZoBell (37) in which lower water temperatures resulted not only in slower degradation rates but also in longer lag periods prior to the onset of detectable biodegradation. Studies of psychrophilic, or at least psychrotolerant, microbes revealed that most oil fractions (except isoprenoids [33] and some cyclics and aromatics [10]) biodegradable at mesophilic temperatures also were readily degraded at the lower temperatures. Microbial populations enriched under psychrophilic temperatures were found to readily metabolize oils of similar quality under mesophilic temperatures, whereas mesophilically-enriched populations exhibited limited activity on similar quality oils under psychrophilic conditions (17, 33).

An interesting interpretation was recently proposed (2, 4) to account for the notably long lag periods preceding biodegradation of oil under psychrophilic conditions. Besides the fairly common lowering of biochemical activity at reduced temperatures, slow evaporation of the aforementioned volatile petroleum components, inhibitory to oil-degrading microorganisms, was credited with retarding biodegradation. At higher (mesophilic) temperatures, the evaporation rate of the inhibitory component(s) was significantly increased, resulting in notably shorter acclimatization periods. "Preweathered" crudes showed little or no lag period.

Induction enrichment temperatures as well as growth temperatures have a marked effect on the generic composition of hydrocarbonoclastic microbes as well as on the degree of biodegradation that can be expected. Enrichment temperatures must be chosen wisely, not only because of their pronounced effect in determining the make-up of the microbial population but also because of the probable wash-out that would occur should the microbes be seeded under incompatible temperatures, i.e., temperatures adverse to enrichment. Although significantly more kinds of microbes grow at mesophilic than at psychrophilic temperatures, the necessity of cultivating psychrophilic or at least psychrotolerant

microbes is substantiated by their ability to degrade most hydrocarbons at higher temperatures. The reverse is not necessarily true. Species must be isolated, however, that will be effective against the more resistant components already mentioned.

TABLE 2. Effect of Temperature on Generic Composition[a] of Microbial Populations Obtained by Enrichment Culture on Oils of Different Chemical Composition[b]

Genus	Oil							
	Prudhoe Bay		Atkinson Point		Norman Wells		Lost Horse Hill	
	4 C	30 C	4 C	30 C	4 C	30 C	4 C	30 C
Achromobacter sp.	22	18	0	27	0	0	0	67
Acinetobacter sp.	0	0	46	0	0	37	5	16
Alcaligenes sp.	22	8	0	34	0	0	50	0
Arthrobacter sp.	0	46	0	3	0	10	2	0
Flavobacterium and *Cytophaga* sp.	53	0	0	0	40	0	0	2
Pseudomonas sp.	0	21	14	0	34	0	41	15
Xanthomonas[c] sp.	0	0	0	39	10	21	0	0
Unidentified Gram negative rods[d]	3	8	0	0	15	64	2	0
Unidentified Gram negative cocci[d]	0	0	40	0	0	0	0	0

[a]After 4 days growth during the fourth transfer.

[b]Adapted from Westlake et al., 1974 (33).

[c]Although these bacteria keyed out as *Xanthomonas* this genus is reserved for plant pathogens; no attempt was made to determine their pathogenicity.

[d]Differing in at least one character from designated genera.

AVAILABILITY OF NUTRIENTS

Potentially a significant drawback in artificially seeding oil spills is the nutrient deficiency of seawater, especially in nitrogen and phosphorus (1, 4, 6, 21, 25, 26). Fairly typical values of total nitrogen and phosphorus in seawater are 980 and 70 µg/l respectively (1). Optimal concentrations of these nutrients for mixed cultures of hydrocarbon degraders were found to be 10 mM nitrate and 0.35 mM phosphate (25). Presumably, these values correspond to the cell surface concentrations of 10^{-7} M nitrogen and 10^{-8} M phosphorus thought to be adequate for microbial growth (26). The preceding figures must be viewed, however, in light of data from artificial seeding experiments

conducted by Miget (21). It was estimated that at least 100 to 1000 times more bacterial cells were concentrated at the oil-water interface than in the seawater itself. The implication is that nutrient salt concentrations at the interface should perhaps be 100 to 1000 times greater than the values typically reported. Methods of maintaining adequate nutrient salt concentrations by periodic spraying and wax encapsulation (21), and application of oleophilic nitrogen and phosphorus "fertilizers" have been considered (4). Although the previously-mentioned techniques have achieved some degree of success, technology for the cheap and effective large-scale addition of phosphorus and nitrogen "fertilizers" must still be developed.

Some comment should be made about not only the addition of nutrient salts to oil spills to facilitate the growth of hydrocarbon degraders but, more generally, the introduction per se of large quantities of microbial cultures into the environment. It is probably fairly safe to say that very few investigations have been conducted to determine the potential pathogenicity of hydrocarbon degraders, or their metabolic intermediates, to humans, animals or to the organisms occurring naturally in the aquatic environment. High concentrations of mixed microbial cultures with presently poorly defined biological activities should be treated with utmost care. Crude oil components (28) and metabolic intermediates and by-products (5, 8) have been shown to adversely affect or even limit naturally-occurring aquatic organisms. To our knowledge, however, the effect of mixed populations of oil degraders on the environment has not been determined. Should artificial seeding of oil spills be judged operationally feasible, screening of the seed organisms should be performed scrupulously. Addition of nitrogen and phosphorus supplements to oil spills will not only enhance the growth of hydrocarbon degraders but also other competitive forms, including pathogenic organisms. Without further information on microbial ecology and microbial species interactions, large-scale addition of nutrient salts should be viewed as a potential and not as a real benefit.

CONCENTRATION OF OIL

Due to the slight solubility of most petroleum hydrocarbons in water, typical concentrations used in investigations of biodegradation have been between 0.2 and 2.0%. Despite the fact that massive concentrations of hydrocarbons may exist in at least local areas of oil spills, the influence of high hydrocarbon concentrations on microbial kinetics and metabolism has been poorly documented. The emulsification of oil could lead to the exclusion of the aqueous medium and local concentrations of oil around the microbes could increase by a factor of several million (26). Qualitatively it has been suggested (36) that at high hydrocarbon concentrations fractions most susceptible to microbial attack would be preferentially degraded, whereas in relatively low concentrations most fractions would undergo at least some degree of degradation.

Although decrease in a physiological parameter (specific growth

rate, oxygen uptake, etc.) of a microbe at high substrate concentrations has generally been associated with substrates not limited by solubility, some hydrocarbon degraders show similar patterns (11, 30, 34). Several mechanisms of substrate inhibition have been summarized by Edwards (12) yet no single inhibition mechanism has been assigned to hydrocarbon degraders. Edwards (12) suggested that inhibition often involves a multiplicity of mechanisms rather than just a single one. The likelihood of an oil micro-environment, rather than an aqueous one, increases with oil concentration and this situation may be the first step leading toward inhibition.

Direct physical contact between microbial cells and the oil substrate probably is required for growth (13, 19, 26). Microorganisms have been shown to adhere to and completely cover oil droplets (13, 19) and even enter and metabolize inside the oil phase, although the degree of oleophilicity varies between microbes (26). A degree of oil emulsification yielding droplets of comparable size to the cells results in an unfavorable configuration for substrate transfer. If small oil droplets adhere to microbial cells, or if the oil droplets are sufficiently large to allow microbial adsorption, growth is enhanced (13).

It has been suggested that formation of water-in-oil emulsions is more common than formation of oil-in-water emulsions and that the former type of emulsion is stabilized by the colloidal asphaltene particles occurring naturally in crudes (27). Coty et al. (9) have conducted fermentations with oil-in-water emulsions and water-in-oil emulsions. They determined that microbes were concentrated in the aqueous phase of water-in-oil emulsions and that the effect of a hydrocarbon layer between air and water accelerated rather than retarded oxygen transfer. The inversion of the oil-phase-continuous condition to one of water-phase-continuous was accomplished by either a dilution of the surfactant used, a drop in the temperature, an increase in the pH or through the production of metabolic intermediates.

The applicability of the preceding reports to seeding of oil spills becomes obvious when comparing the susceptibility to wash-out of the microorganisms trapped in the aqueous phase of the water-in-oil emulsion with the microorganisms concentrated at the interface of the water and oil phases. It might be suggested that the varied oleophilic nature of hydrocarbon oxidizers, as well as the genetic capability of producing extracellular emulsifiers, determine where or why a particular microbe is concentrated in a particular type of oil waste or spill.

The large increase in production of cellular and extracellular lipids by *Acinetobacter* sp. as a result of hydrocarbon metabolism has been demonstrated by Finnerty et al. (15). Although extracellular excretions are thought to increase oil emulsification (35), the reason for this rather dramatic metabolic reaction and the extent to which other microbes may be influenced in a similar way remain unknown. The study by Finnerty et al. (15) also demonstrated the distorted metabolism and internal structure of *Acinetobacter* when grown on hexadecane. Electron micrographs showed internal hydrocarbon inclusion bodies;

similar changes have been demonstrated for *Candida lipolytica* (23) and *Torulopsis* sp. (19) as well. *Acinetobacter* cells cultivated on hexadecane occasionally grew to 4 to 10 times their normal size, becoming greatly extended and elongated with extensive intracytoplasmic membrane development. The development of the hydrocarbon inclusions is significant because it was typical of hydrocarbonoclastic bacteria as well as yeasts and filamentous fungi. In the case of *Candida lipolytica*, the organism was unable to distinguish between utilizable and non-utilizable hydrocarbons; both types of hydrocarbons were found within the cell (23).

It is perhaps important to question just how far the included hydrocarbons may travel in the food chain before disappearing, before being converted to non-toxic intermediates or before being metabolized to potentially harmful ones. To our knowledge, no explanation has been proposed to interpret how or why a microorganism stores hydrophobic hydrocarbons against a concentration gradient or how or why it undergoes significant cellular distortions. Correlations between high hydrocarbon concentrations, cellular transformation, hydrocarbon inclusions, and their subsequent effects on the aquatic environment are only tentative as no definitive studies are available.

DECLINE OF NON-MARINE MICROORGANISMS

It is quite probable that seawater and the open ocean provides the impetus for another form of microbial selective enrichment. The saline nature of seawater is a first consideration. Many microbes originally isolated from the sea have obligate salinity requirements which must be satisfied in preparation of growth media. For other microorganisms, natural seawater is inhibitory or even toxic, the effect of salinity being only one factor. A number of factors affect the decline of microorganisms, particularly non-marine organisms, in seawater. These have been summarized in a review paper by Mitchell (22).

Several studies have indicated that a biological heat-labile toxin produced by marine microbes was the bactericidal agent in seawater. This factor was either diminished or eliminated by boiling, autoclaving, filtration, pasteurization or chlorination. A number of species of marine bacteria and actinomycetes were tested for their antagonism toward non-marine microbes. A substantial proportion (9 of 58) were antagonistic to non-marine microorganisms and it was thought that the antagonistic material was an antibiotic. It was also found that some marine bacteria were able to lyse the cell wall of non-marine microbes. Other naturally-occurring sea organisms were active against microbes either through a parasitic action or directly through predatory activity. The native marine microflora was also found to competitively displace non-marine microorganisms under the growth-limiting conditions in the sea. The removal of microbes by physical means such as adsorption, flocculation and sedimentation was also thought to be prevalent.

The possibility of oil spills occurring in either salt or fresh water areas probably requires that enrichment cultures of either marine

or non-marine types of microorganisms be available. Salinity per se is not the only factor that affects the decline of the non-autochthonous (non-native) microorganisms in the sea but that certain biological, chemical and physical characteristics of the oceans are important factors in the decline of certain microbial populations.

CONCLUSIONS

An oil spill acts as a selective enrichment system to continuously select for microorganisms capable of causing degradation and eventual break-up of the oil. In some spills, seeding with microorganisms may not be necessary because of the presence of indigenous hydrocarbonoclastic microbes. Data would indicate that indigenous populations can remove paraffinics, whereas the more complex hydrocarbons are removed by highly specific microbes. However, dictates for seeding are probably more appropriately governed by: (1) the harshness of the environment, e.g., saline condition of the ocean, and temperatures as low as 4-5 C; (2) highly toxic components present in oil, e.g., phenols, aromatics, carcinogenic compounds, etc.; (3) nutritional condition of the environment, i.e., adequate inorganic nitrogen and phosphorus to support growth of either indigenous populations or special seed cultures. If the environment per se imposes constraints, the application of specially adapted and mutated cultures is necessary. Likewise, if the oil spill contains toxic components which are normally inhibitory to most microbes, specially adapted and mutated cultures may be far more beneficial than the indigenous populations.

Selective continuous enrichment systems are easily studied in the laboratory. This technique is adaptable for selecting mixed microbial populations which can compete under adverse chemical and physical conditions of the environment. Thus, data presented herein suggest that microbial seeding of oil spills in aqueous environments may be beneficial. Nevertheless, adequate studies to establish limitations and instances of where biotreatment would be most successful are required.

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PETROLEUM BIODEGRADATION IN THE ARCTIC

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ABSTRACT

Alaskan Arctic waters from Prudhoe Bay were found to contain indigenous microbial populations, capable of degrading petroleum hydrocarbons, in concentrations similar to those found in our prior studies of temperate Atlantic coastal waters. The microorganisms were capable of degrading Prudhoe crude oil at 5 C and showed greater oil emulsification than previously studied organisms. Southern Alaskan waters from Port Valdez also contained oil-degrading microorganisms, but these degraded Prudhoe crude at much slower rates than the Arctic organisms and did not show the same ability to emulsify oil. In situ tests in Prudhoe Bay revealed higher oil biodegradation rates when a N and P fertilizer was added. Increases of several orders of magnitude in the populations of oil degraders, especially *Pseudomonas* species, were found underlying miniature oil slicks in Prudhoe Bay. These increases were accompanied by a similar, although slightly less extensive, increase of *Staphylococcus epidermidis*, a non-oil degrading mesophile. Several oil degrading bacteria and yeasts associated with naturally-occurring seepages, or artificially-introduced oil spills, in the Arctic were isolated. These organisms, including members of the genera *Rhodotorula* and *Pseudomonas*, are being tested as possible seed inocula to enhance oil biodegradation in the Arctic.

INTRODUCTION

The discovery and plans for utilization of Alaska's north slope petroleum resources has raised questions of concern over the fate of accidentally spilt oil. The adverse environmental conditions that characterize the Arctic usually preclude physical removal of the oil, leaving any spilt oil to natural degradative and dispersal processes, including emulsification, evaporation of the volatile fraction, microbial degradation, and deposition of the undegraded residue.

The non-biological weathering and dispersal of petroleum is largely a function of its chemical nature and environmental factors such as temperature, wind, and wave action. The natural biodegradative rates of

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petroleum in the sea are usually limited by low numbers of microorganisms, scarcity of essential nutrients, and low temperature (7). Low concentrations of nitrogen and phosphorus in sea water have been shown to prevent extensive petroleum biodegradation (2). In the Arctic, temperature is expected to have a profound limiting influence on microbial oil-degrading activities. In addition to reducing rates of microbial metabolism, low temperatures limit evaporation of toxic components of at least one crude oil, delaying its biodegradation (5). Nevertheless, some microorganisms certainly are capable of degrading petroleum at low temperatures (3, 1, 16, 13, 10). One might expect such organisms to be absent or in very low concentrations in pristine Alaskan coastal waters. Robertson et al. (10) reported, however, that such organisms are widely distributed in the Gulf of Alaska and are present, but more difficult to isolate, in the Arctic. ZoBell (16) also has reported that microorganisms from the Arctic can extensively degrade petroleum hydrocarbons and crude oils at sub-zero temperatures, including up to 61% of Prudhoe crude within 10 weeks at -1.1 C. The distribution and in situ activity of such organisms in the Arctic, however, has not been determined previously.

The present study was undertaken to determine natural rates of petroleum degradation in Alaskan coastal waters, to elucidate the factors that limit these degradative processes, and to explore methods for overcoming these limitations. The first aim was to determine what would happen if Prudhoe crude oil accidentally entered these waters; whether there were sufficient microorganisms, adequate nutrient levels, and suitable temperatures to permit rapid microbial degradation of this oil. The next objective was to examine possible ways of accelerating these degradation rates and possible benefits of "seeding" and fertilizing the oil slicks with suitable nutrients and organisms. To this end we tested the effects of addition of an oleophilic fertilizer previously developed (6). We also isolated a number of potential "seed" organisms associated with natural Arctic oil seepages or that were enriched for underlying artificially introduced slicks of Prudhoe crude. It was felt that autochthonous organisms would be best for seeding, providing that the numbers of extant oil-degrading microorganisms was a limiting factor.

MATERIALS AND METHODS

SAMPLING PROCEDURES

Water samples were collected from Prudhoe Bay and Port Valdez, the proposed terminals of the Trans-Alaskan pipeline (Fig. 1). Prudhoe Bay is very shallow, with an average depth of 0.61-1.22 m (15), and is completely frozen over most of the year, being ice-free for only 1-2 months. Port Valdez is much deeper, with an average depth of over 30 m and is open year round (14). Surface samples were collected in sterile Erlenmeyer flasks. Bottom samples were collected using a Van Dorn Water Sampler (Hydro Products, San Diego, Calif.). The bottle was rinsed several times with bottom water before a collection. The samples were

stored on ice and returned to the laboratory for analysis.

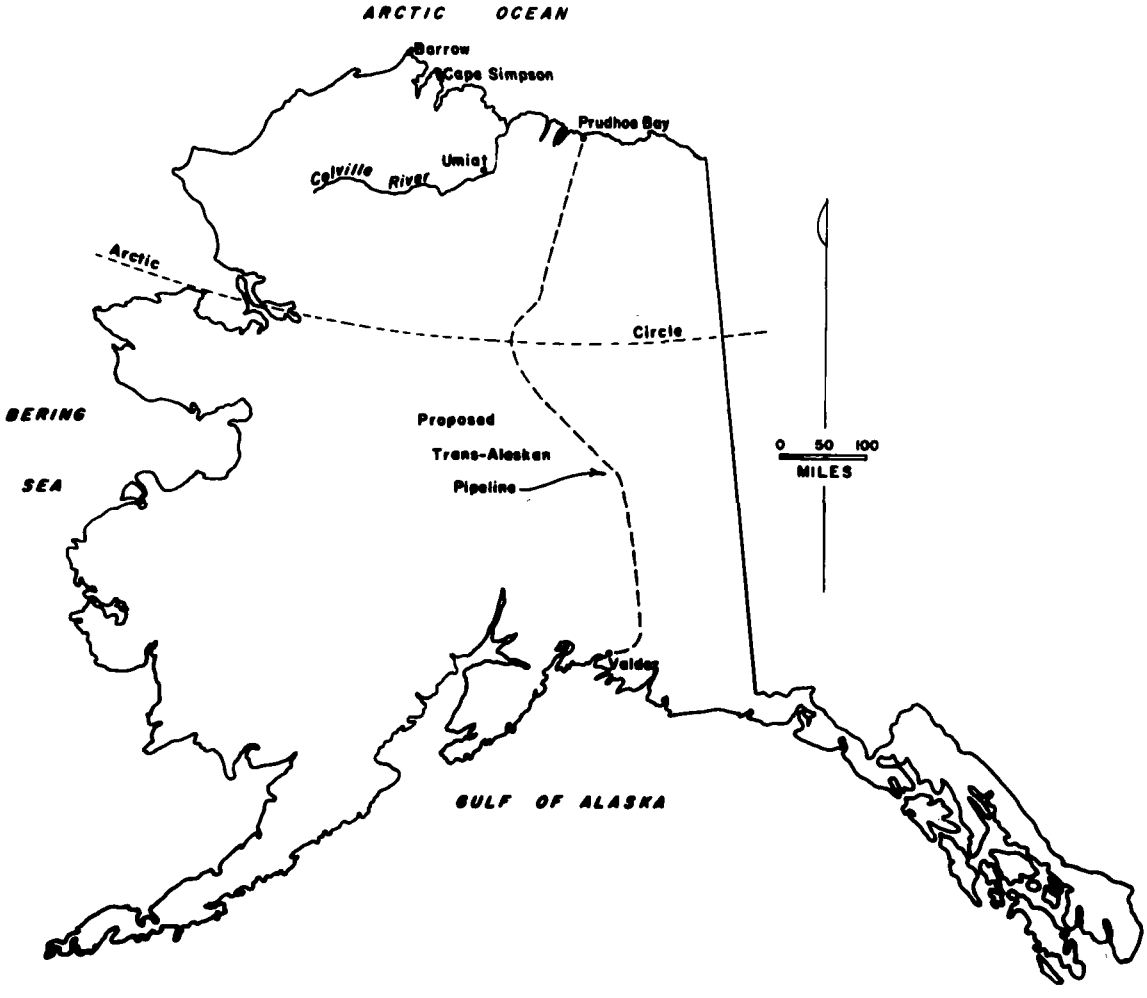

FIGURE 1. Map of Alaska showing proposed Trans-Alaskan Pipeline route.

Water, soil, and oil samples were also collected from natural oil seepages at Cape Simpson, seep 2 (11) and Umiat (8), using sterile whirl-pack bags. Samples from the Cape Simpson seep were collected along a traverse from one end of the seep downhill to a lake where the seep ended. Samples were taken from the surfaces, both inside the seep, including oil, tar and overlying pools of water, and from the adjacent soil and water. Samples from the Umiat seep were obtained from soil at the surface overlying the seep at several depths down to the permafrost, 46 cm.

WATER ANALYSIS

Water temperature and salinity were measured at each sampling site using a model RS-5 salinometer (Beckman Instruments, Inc.). Salinity also was checked on samples returned to the laboratory using a model RS-7a inductive salinometer (Beckman Instruments, Inc.). Oxygen concentration was measured with a dissolved oxygen meter (Yellow Springs Instrument Co., Yellow Springs, Ohio). The pH was measured with a pH meter (Beckman Instruments, Inc.). Nitrogen, in the form of ammonium ions or nitrate plus nitrite ions, total phosphate, and iron concentrations were determined using a Hach water tester (Hach Co., Ames, Iowa). Nitrate and ammonium ion concentrations were also measured using a Technicon Autoanalyser (analyses courtesy of Dr. R. Horner).

ENUMERATION OF MICROORGANISMS

Total viable counts were performed using either marine agar 2216 (Difco) for Bay and sea water samples, or nutrient agar (Difco) for coastal pond and soil samples. For fungi, Dextrose-Sabouraud agar (Difco) was used. All samples were stored on ice between collection and time of plating, a period not exceeding 24 hr. Serial dilutions of water or soil samples were surface-spread onto the agar in petri plates using a Drigalsky spatula, and incubated at 5 or 25 C. Colonies that developed were enumerated at 7 and 14 days using a New Brunswick Scientific Co. colony counter (12). Oil-degrading microorganisms were tabulated by passing 100 ml water samples through a 0.45 µm porosity membrane filter (Millipore Filter Corp.), the latter than plated on an oil-agar medium, as previously described (5). Colonies that developed after incubation at either 5 or 25 C for 14 or 28 days were counted and isolated for later identification and determination of their hydrocarbonoclastic ability.

CRUDE OIL BIODEGRADATION POTENTIAL

One hundred milliliter aliquots of water samples from Port Valdez or Prudhoe Bay were placed in a gas train arrangement similar to one previously described (4), but the flasks were not shaken. One milliliter of either Prudhoe crude oil (gift of Atlantic Richfield Co.) or *n*-hexadecane (Aldrich Chemical Co.) and one ml of a stock solution of phosphate and nitrate were added, in final concentrations of 0.5 mM and 10 mM, respectively. Incubation was at 25 C with constant aeration with CO_2-free air. CO_2 evolution from hydrocarbon degradation was cumulatively monitored. After 42 days of incubation, the residual oil of *n*-hexadecane was recovered by continuous extraction for 12 hr with diethyl ether in a liquid-liquid extractor (Kontes Glass Co.).

Fifty milliliter aliquots of the water samples were also placed into biometer flasks (Bellco Glass Co.). One-half milliliter of either Prudhoe crude oil or *n*-hexadecane and 0.5 ml of the nitrate and phosphate stock solution described above were added, with incubation at 5 or 25 C. CO_2 evolution was monitored cumulatively. After 35 days, the

residual hydrocarbon was extracted as described above. The amount of residual crude oil or *n*-hexadecane was determined gravimetrically.

For isolated microorganisms, a 48-hr culture from either marine broth 2216 or nutrient broth was centrifuged (3500 g for 15 min), washed twice in and resuspended in Bushnell Haas broth. All broths used were Difco products. One milliliter containing 1.0 mg protein was inoculated into 250 ml Erlenmeyer flasks containing 99 ml sterile Bushnell Haas broth and one ml of either *n*-hexadecane, pristane, Sweden crude (Sun Oil Co.) or Prudhoe crude (Atlantic Richfield Co.). The flasks were incubated at 5 C with rotary shaking, 240 rpm for 30 days. The residual hydrocarbons were recovered by extraction with two 50 ml portions of diethyl ether. The extracts were analyzed gravimetrically and by quantitative gas liquid chromatography using a Hewlett Packard model 5700 flame ionization detector gas chromatograph with dual 3 m O.D. by 1.8 mm long columns packed with 10% Apieyon L on 60/80 mesh Chromosorb (4).

IN SITU CRUDE OIL BIODEGRADATION

A styrofoam float containing Plexiglas cylinders, similar to one previously described (6), was anchored in Prudhoe Bay at the mouth of the Putuligayuk River (Fig. 2, site 2). The Plexiglas cylinders were 5 cm in diameter and 46 cm in length. One milliliter Prudhoe crude oil was added to each cylinder. To some of these miniature oil slicks an oleophilic nitrogen and phosphorus fertilizer (6) also was added. For other control slicks, the bottoms of the cylinders were stoppered and 1% $HgCl_2$ added. The float was set out in mid-June but constant shifting of the sea ice pushed the apparatus onshore or upset the tubes and it was early July before the float was continuously deployed. A second float set out near the Prudhoe dock (Fig. 2, site 1) was totally destroyed by the ice. Replicate cylinders with their crude oil contents were removed periodically until mid-August. The microbial populations in the water columns underlying the oil slicks were enumerated as described earlier. Selected microorganisms were isolated and their oil biodegradation potential determined. The oil was recovered from the removed cylinders by continuous extraction for 24 hrs using liquid-liquid extraction and diethyl ether as solvent. The residual oil was measured gravimetrically.

RESULTS

WATER ANALYSES

The properties of the water samples collected in mid-June at two locations in Port Valdez are shown in Table 1. Site one was adjacent to the proposed Trans-Alaskan pipeline terminus; site two was at Valdez Narrows. The surface waters were relatively warm and well aerated. Both sites had comparable nutrient concentrations although the salinity at site two was significantly higher (Table 1).

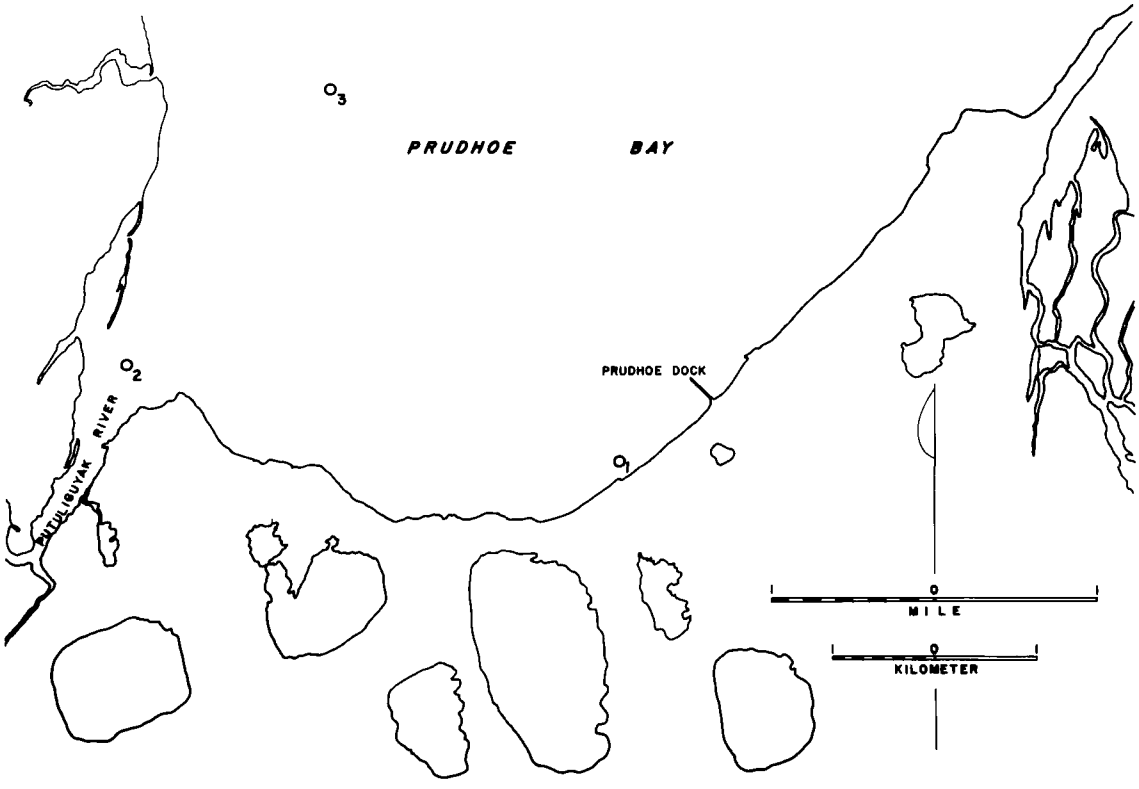

FIGURE 2. Map of Prudhoe Bay showing sampling locations.

Table 1. PROPERTIES OF WATER SAMPLES COLLECTED AT PORT VALDEZ

Property	Location	
	Proposed southern pipeline terminus	Valdez Narrows
Temperature	11 C	12 C
Salinity	9‰	16.4‰
Oxygen concentration	saturated	saturated
$PO_4^{\equiv}$	0.14 ppm	0.11 ppm
N as NH_4^+	1.0 ppm	1.3 ppm
N as NO_3^- + NO_2^-	0.08 ppm	0.08 ppm
Fe	0.1 ppm	0.08 ppm

Water samples also were collected at several sites in Prudhoe Bay from late June to early August. Three sites were sampled (Fig. 2), one about 0.8 km west of Prudhoe dock (site 1), a second at the mouth of the Putuligayuk River (site 2), and a third about 3.2 km north of the river mouth, where the water depth was 2.13 m (site 3). The properties of these water samples are shown in Table 2. As noted, until July 15 the Bay was highly stratified, with a bottom layer of cold, saline, nutrient-deficient water. The thermocline was at about the 1.82 km level in the deeper portions of the Bay. Until late July, there was considerable ice in the Bay and water derived from its melting would be nutrient-deficient and of low salinity. The constant sunlight probably explains the relatively high surface water temperature. It should be noted that, between the July 15 and July 22 samplings, a storm passed through Prudhoe Bay, apparently turning over the Bay, as evidenced by the lack of a thermocline on the July 22 sample shown by the similar surface and bottom temperatures and salinities on that date.

ENUMERATION OF MICROORGANISMS IN ALASKAN COASTAL WATERS

The microbial populations in the Port Valdez and Prudhoe Bay samples are shown in Table 3. The Prudhoe Bay populations were somewhat higher than those found in Port Valdez. A notable population was capable of growth at 5 C, especially at Prudhoe Bay. The total populations in Prudhoe Bay showed a general decline until after the Bay had turned over and the ice had disappeared. Enumeration of fungi at 25 C showed concentrations varying between 0 and 10 viable units/ml. No fungi were isolated when incubation was at 5 C.

The numbers of oil degraders in the surface waters, enumerated at 25 C, were about 100 cells/L at Valdez and 700 cells/L in Prudhoe Bay. The microbial oil-degrading population in Prudhoe Bay showed only minor fluctuations in size over the summer. The oil-degrading population in Prudhoe Bay enumerated at 5 C was only slightly lower, about 600 cells/L.

NATURAL OIL SEEP AREAS

The Cape Simpson oil seep No. 2 is an extensive natural seep consisting of older black asphaltic material and fresher soft red-white-brown oil. At the time of sampling, the temperature of the soil and water associated with the seep was about 10 C and the pH 5.7. The soft oil-water emulsion contained only fungi, one pink-pigmented and one cream-colored yeast and one filamentous green-black imperfect fungus tentatively identified as a *Rhodotorula* sp., a *Candida* sp. and a *Mucor* sp., respectively. The size of this fungal population was 4×10^6 viable units/g and 1×10^3 viable units/g enumerated at 25 and 5 C, respectively. The adjacent soil had 3×10^6 bacteria/g and 4×10^4 fungi/g enumerated at 25 C. An adjacent lake not directly in contact with oil had 2×10^5 bacteria/ml and 3×10^2 fungi/ml. The lack of bacteria in the seep is unknown, but the low pH may be a contributing factor.

Table 2. PROPERTIES OF WATER SAMPLES COLLECTED IN PRUDHOE BAY

Property	Location and Date																	
	Site 1 surface				Site 2 surface					Site 2 bottom[a]			Site 3 surface			Site 3 bottom[b]		
	6/28	7/8	7/15	7/22	6/28	7/8	7/15	7/22	8/8	7/8	7/15	7.22	7/8	7/15	7/22	7/8	7/15	7/22
Temperature (C)	7.7	8.3	11.0	4.5	3.3	8.4	11.8	4.9	7.5	8.2	11.3	4.9	7.7	7.9	4.3	-1.1	-1.0	4.3
Salinity (‰)	3.5	5.2	5.1	11.5	7.6	3.2	2.8	10.0	7.9	4.0	4.1	10.0	7.5	7.4	11.6	67.8	67.5	12.0
Oxygen concentration	sat.	ND[c]	ND	sat.	sat.	sat.	ND	sat.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
$PO_4^{\equiv}$ (ppm)	ND	0.05	0.05	0.09	ND	0.05	0.05	0.10	ND	0.05	0.05	0.08	0.05	0.04	0.11	0.23	0.21	0.15
N as NH_4^+ (ppm)	ND	0.05	0.06	0.93	ND	0.05	0.04	0.15	ND	0.15	0.18	0.23	0.17	0.15	0.69	2.2	2.3	0.75
N as $NO_2^- + NO_3^-$ (ppm)	ND	0.03	0.03	0.03	ND	0.06	0.03	0.09	ND	0.02	0.02	0.04	0.05	0.03	0.05	0.4	0.4	0.5

[a] 0.914 m [b] 2.13 m [c] ND = not done

Table 3. TOTAL MICROBIAL POPULATIONS IN ALASKAN COASTAL WATERS

Date	Valdez				Prudhoe Bay									
	Pipeline terminus		Narrows		Site 1 surface		Site 2 surface		Site 2 bottom		Site 3 surface		Site 3 bottom	
	5 C	25 C	5 C	25 C	5 C	25 C	5 C	25 C	5 C	25 C	5 C	25 C	5 C	25 C
	Number of Viable Cells (in hundreds/ml)													
6/12	1.2	3.1	1.0	1.9	--	--	--	--	--	--	--	--	--	--
6/28	--	--	--	--	270	630	2	1100	--	--	--	--	--	--
7/8	--	--	--	--	3.6	510	1.5	500	--	--	--	--	--	--
7/15	--	--	--	--	4.0	23	2.8	21	17.1	18	7.0	20	10	70
7/22	--	--	--	--	53	61	62	140	11.2	132	29	69	33	73
8/8	--	--	--	--	--	--	130	420	--	--	--	--	--	--

The seep at Umiat was much smaller than the Cape Simpson seep. The surface soil was largely root mat with a temperature of 10 C. Just below the organic root mat was a clay layer 1 C at the top and 0 C at 30.5-46 cm. The microbial concentrations at Umiat were much higher than in the more northern locations. There were 3×10^9 bacteria/g in the surface soil and 2×10^8 bacteria/g in the subsurface soil at 30.5 cm, enumerated at 25 C, and 5×10^8 bacteria/g surface soil and 4×10^8 bacteria/g subsurface soil enumerated at 5 C. The fungal populations were 2×10^6/g and 4×10^4/g for the surface and 5×10^3/g and 6×10^2/g for the subsurface (30.5 cm) at 25 and 5 C, respectively.

CRUDE OIL BIODEGRADATION POTENTIAL

The potential of the indigenous microbial populations of Port Valdez and Prudhoe Bay to degrade hydrocarbons was determined in several experiments. When Prudhoe crude oil or *n*-hexadecane was incubated in gas train experiments at 25 C, the Prudhoe Bay water showed a higher rate of mineralization than the Valdez water with a greater CO_2 production from *n*-hexadecane than from Prudhoe crude (Fig. 3). The rates of CO_2 evolution were diminished when incubation was in biometer flasks where there was no aeration or agitation, but the same relationship existed between Prudhoe Bay vs. Port Valdez water and *n*-hexadecane vs. Prudhoe crude (Figs. 4 and 5). The rates of hydrocarbon mineralization (CO_2 production) were reduced at 5 C but no extensive lag periods were noted before the onset of mineralization. The percent biodegradation after 31 days of incubation was consistent with the relative amounts of CO_2 evolved (Table 4). The non-biological losses, determined with sterile controls, were 1 and 5% at 5 C, and 11 and 23% at 25 C of the added *n*-hexadecane and Prudhoe crude oil, respectively. There was also a qualitative difference between the biodegradation of Prudhoe crude by the microorganisms in Port Valdez and Prudhoe Bay. The entire water column turned brown with Prudhoe Bay water, indicating extensive emulsification which did not occur with the Valdez water. The chemical explanation for this observation is not yet known.

Table 4. PERCENT BIODEGRADATION OF *n*-HEXADECANE AND PRUDHOE CRUDE OIL BY THE INDIGENOUS MICROORGANISMS IN PORT VALDEZ AND PRUDHOE BAY DURING 31 DAYS OF INCUBATION

	Percent oil biodegraded			
	Port Valdez		Prudhoe Bay	
	5 C	25 C	5 C	25 C
Prudhoe crude	7	26	21	39
n-hexadecane	26	32	41	57

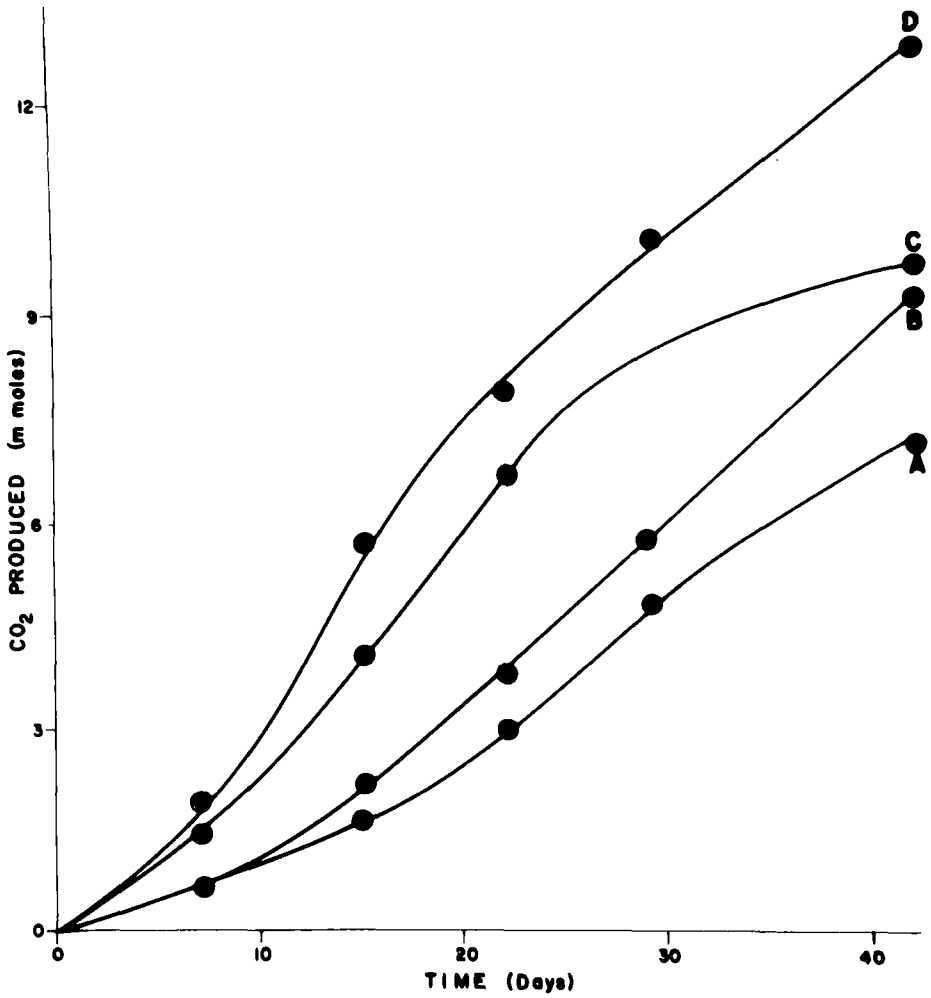

FIGURE 3. Mineralization of petroleum hydrocarbons by indigenous microbial populations of Alaskan coastal waters at 25 C with aeration. A = Port Valdez--Prudhoe crude. B = Port Valdez--*n*-hexadecane. C = Prudhoe Bay--Prudhoe crude. D = Prudhoe Bay--*n*-hexadecane.

The ability of the isolated potential seed organisms to degrade hydrocarbons varied from none to extensive. Apparently some organisms associated with crude oil enrichment develop via a commensal relationship. Two *Pseudomonas* species, one isolated from the Umiat seep and the other from Prudhoe Bay, showed the greatest oil degradation potential. Both organisms were able to degrade about 0.5 g Prudhoe crude oil at 5 C within 4 weeks, and were able to attack resistant components such as pristane as well as the more easily degraded *n*-paraffins. The fungi isolated from the Cape Simpson seep showed only limited crude oil degradation, the range of hydrocarbons subject to attack being limited to *n*-paraffins. Even this latter activity was negligible at low temperatures.

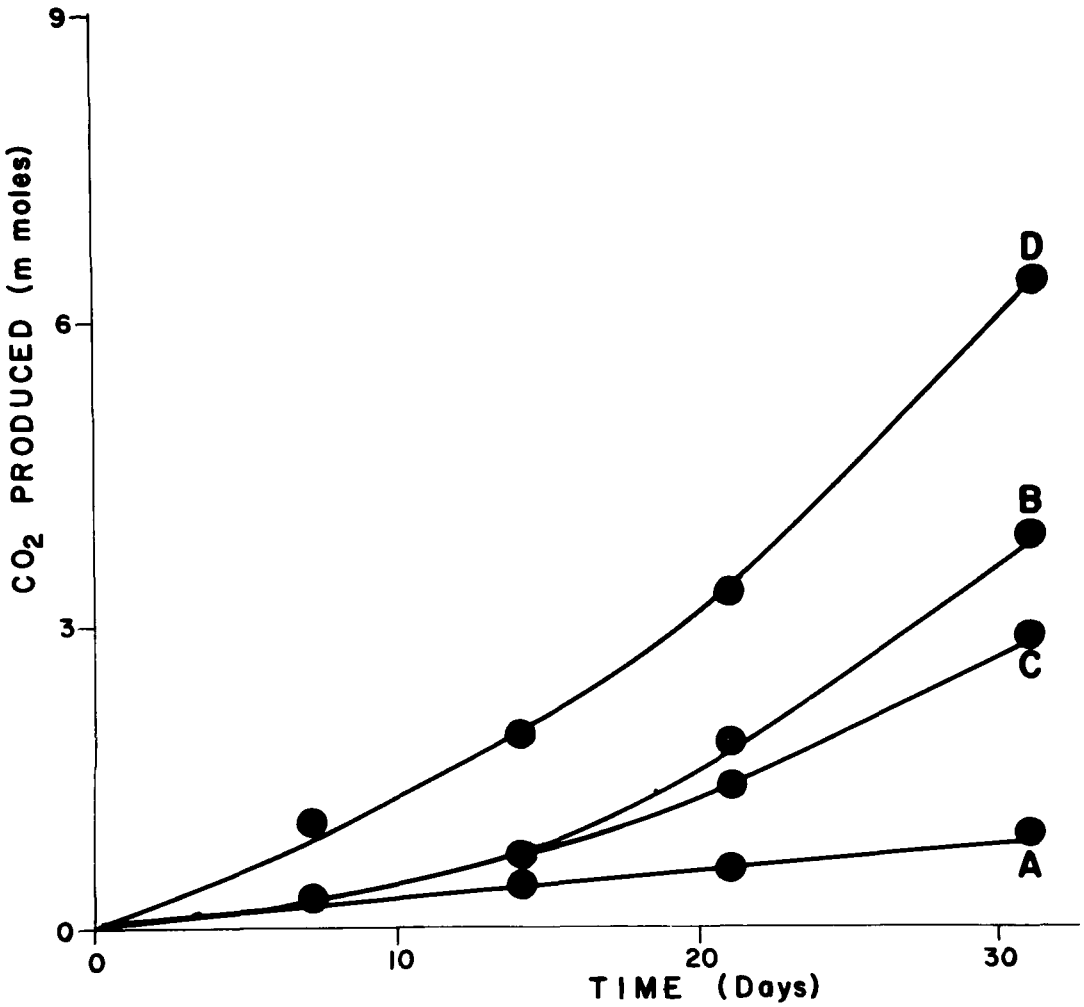

FIGURE 4. Mineralization of Prudhoe crude without aeration.
A = Port Valdez--5 C. B = Port Valdez--25 C.
C = Prudhoe Bay--5 C. D = Prudhoe Bay--25 C.

IN SITU CRUDE OIL BIODEGRADATION:
NATURAL AND FERTILIZER-STIMULATED

The percent oil lost from miniature slicks floated in Prudhoe Bay is shown in Fig. 5. The float was located at Prudhoe Bay site 2. Tables 2 and 3 show the physico-chemical and microbiological properties of the water. The greatest extent of biodegradation occurred when the oleophilic N and P fertilizer was added. The apparent biodegradation losses may be somewhat inflated due to extensive emulsification which was observed in the in vitro studies. The microbial populations in the water columns underlying the slicks are shown in Table 5. There was a great increase in the populations underlying the active slicks, with the largest increase under the fertilized slicks. The elevated microbial biomass reflects the increase of a non-pigmented, gram negative,

short, rod-shaped bacteria and a gram positive coccus. The normal diverse population, including highly pigmented organisms, appeared to remain in the same total numbers as in the water column adjacent to the float. The organisms showing the large population increases have been identified as a *Pseudomonas* sp. and *Staphylococcus epidermidis*. When tested for their ability to degrade petroleum hydrocarbons, only the *Pseudomonas* sp. gave positive results. This organism was able to extensively degrade Prudhoe crude at both 5 and 25 C. The *Staphylococcus* was unable to attack hydrocarbons at either 5 or 25 C and failed to grow at 5 C. It is not yet known whether this bacterium can grow at the expense of intermediary hydrocarbon metabolites which would explain its increase.

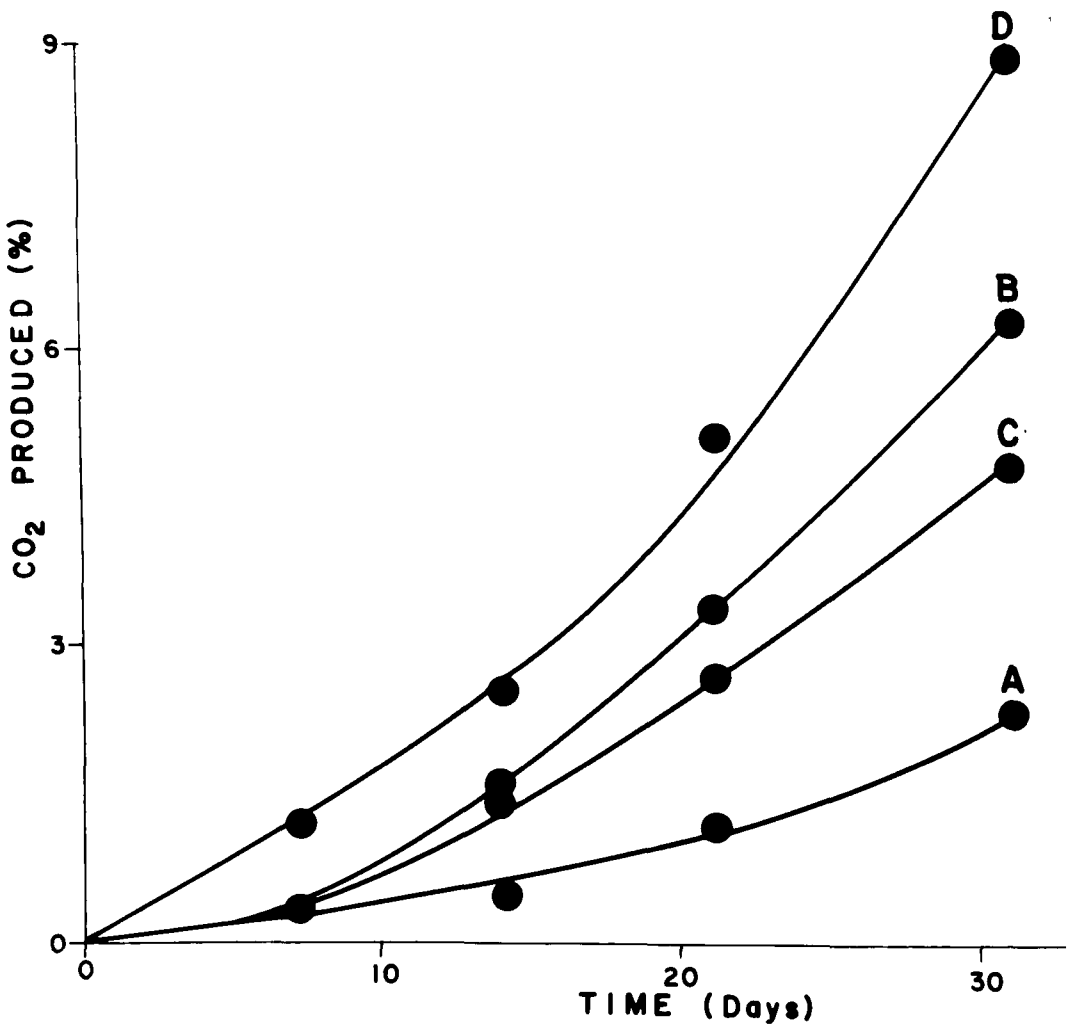

FIGURE 5. Mineralization of *n*-hexadecane without aeration.
A = Port Valdez--5 C. B = Port Valdez--25 C.
C = Prudhoe Bay--5 C. D = Prudhoe Bay--25 C.

Table 5. MICROBIAL POPULATIONS IN THE WATER COLUMNS UNDERLYING THE CONTAINED OIL SLICKS IN PRUDHOE BAY

Date	Unfertilized slick	Fertilized slick	Adjacent water column
	Number of viable cells (in hundreds/ml)[a]		
6/28	1,100	1,100	1,100
7/8	690	760	500
7/15	370	5,300	21
7/22	400	20,000	140

[a]At 25 C.

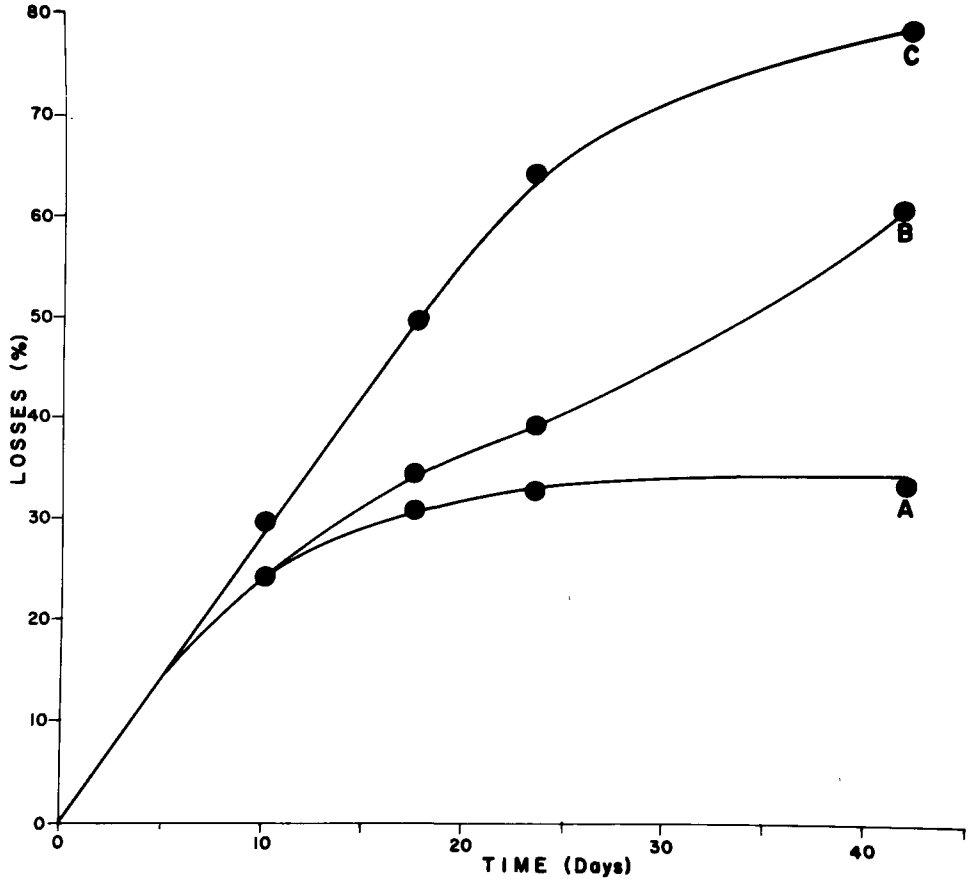

FIGURE 6. In situ losses of Prudhoe crude oil in Prudhoe Bay.
A = non-biological (poisoned). B = natural.
C = stimulated (fertilized)

DISCUSSION

Prudhoe crude, the type of crude oil found in the Prudhoe Bay region, is a relatively low aromatic, high paraffinic oil with a moderate sulfur content, about 0.9%, and a notable lack of light hydrocarbons, C_3-C_6 (9). Since this is the type of crude which is likely to accidentally enter the environment at the Trans-Alaskan pipeline terminals or along the shipping routes from Prudhoe Bay to the conterminous states, the fate of this crude was chosen for study. Similarly, Prudhoe Bay and Port Valdez, the most likely locations for spillages, were selected for intensive study. Two rivers, the Putuligayuk and the Sagavanirktok, flow into Prudhoe Bay and conceivably could carry oil spilt inland into the Bay.

The surface temperatures during the summer in both Prudhoe Bay and Port Valdez were sufficiently warm for microbial oil degradation. The laboratory experiments showed slower rates at 5 C compared with 25 C, nevertheless, an active microbial oil-degrading population was present at the lower temperature. No extensive lag periods were found before the onset of Prudhoe crude biodegradation, as had been observed earlier for Sweden crude (3), indicating a lack of a volatile toxic fraction in the Prudhoe oil. The missing light fraction in Prudhoe crude possibly explains this observation and eliminates the need to consider removal of this fraction by measures such as ignition in order to stimulate biodegradation.

Prudhoe Bay was highly stratified, indicating little mixing even through the shallow water column. This lack of mixing, coupled with the relatively low nutrient concentrations, suggests that fertilization probably would be essential for extensive biodegradation. In situ oil biodegradation experiments, however, did show high losses from the unfertilized slicks. It is not known whether all of these losses represent emulsified but undegraded oil. The latter is a likely possibility in view of the extensive emulsification observed in enclosed laboratory studies using Prudhoe crude and Prudhoe Bay water. Fertilization with an oleophilic nitrogen and phosphorus source clearly enhanced biodegradation but the losses from the fertilized slicks may also be inflated, particularly in light of the lower degradation rates in laboratory experiments compared with those in the field.

In spite of the harsh environmental conditions, the microbial populations in Prudhoe Bay were high, being surprisingly higher than in Port Valdez, and comparable to those found in temperate waters such as Raritan Bay, New Jersey (7). Thus, there was an adequate oil-degrading microbial population in these Alaskan waters. The size of this microbial population increased when exposed to Prudhoe crude, especially under nutritionally favorable conditions. Introduction of these oil-degrading organisms in high numbers, by "seeding," may enhance oil removal by eliminating the lag period necessary for the population to naturally increase to concentrations capable of extensive oil biodegradation. Such seeding with oil-degrading microorganisms might be especially useful in

Port Valdez where the microorganisms are not adapted to Prudhoe crude and were found in our studies to be less capable of degrading oil pollutants. Such seeding would shorten the time course of natural degradation and would not be expected to alter the extent of oil degradation. Re-inoculation with the same organisms that would naturally "bloom" following an oil spillage should not result in any additional environmental damage.

Several possible seed organisms were isolated from Prudhoe Bay and natural Arctic oil seeps. Natural oil seepages especially were examined for oil utilizers. Effective oil degraders also were isolated from Prudhoe Bay. The potential advantage of seeding with these organisms must be demonstrated in future field experiments. Also to be examined is the fate of oil spilt during the winter, when Prudhoe Bay is frozen, and whether oil biodegradation can be stimulated under the harsh conditions of the Arctic winter.

ACKNOWLEDGMENT

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EFFECTS OF HYDROCARBONOCLASTIC YEASTS ON POLLUTANT OIL AND THE ENVIRONMENT*

Nancy H. Berner, Donald G. Ahearn, and Warren L. Cook**

ABSTRACT

The use of hydrocarbonoclastic microorganisms has been recommended for facilitating the removal of residual hydrocarbons from shipboard and harbor bunkers and from natural sites polluted with oils. The degree of microbial emulsification and utilization of oil varies markedly with the type of oil and fluctuating environmental conditions. Mixed or pure cultures able to utilize significant amounts of the asphaltene fractions of oil have not been obtained. Moreover, microorganisms with the capacity to emulsify appreciable amounts of oil have yet to be proven of practical value under field conditions. Only cursory attention has been given to the potential toxicity of the organisms, including the by-products of growth on oil and the possible pathogenicity of the cultures for flora or fauna in the environment.

Two hydrocarbonoclastic yeasts with emulsifying properties, *Candida lipolytica* 37-1 and *C. subtropicalis* R42, were seeded into fresh water estuarine environments inundated with a light, high-paraffinic Louisiana crude oil. Significantly higher populations of predator species (i.e., various protozoa and nematodes and, in the estuarine site, gastropoda) were observed at yeast-seeded sites. *Candida lipolytica* was not recovered from seeded sites after 3 to 5 months, whereas *C. subtropicalis* persisted for over a year in fresh water systems and over seven months in estuarine systems. At the estuarine site, the seeded yeasts eventually were replaced by indigenous flora, some of which after continued exposure to crude oil apparently acquired the ability to utilize hydrocarbons.

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INTRODUCTION

The capacity to metabolize various hydrocarbons has been demonstrated for a diversity of microorganisms. One or more species of seventy genera, including twenty-eight bacteria, thirty filamentous fungi, and twelve yeasts, have been shown to oxidize one or more kinds of hydrocarbons (47). Hydrocarbon-metabolizing microorganisms have been isolated from air, soil, water, animal feces, food products, and other sources (4, 15, 16, 18, 24, 40), but they have been found in greater numbers in environments associated with oils (7, 26, 27).

The *n*-alkane fraction of petroleum has, in laboratory experiments, been biodegraded most completely. Alkanes in the range of kerosene, C_{10} to C_{18}, are utilized more readily than those in the range of gasoline, C_5 to C_9 (20). Crude oils or refinery distillates with a high asphaltic content are less biodegradable than those with an abundance of aliphatic hydrocarbons, and generally are assimilated by a limited number of microorganisms (7).

Numerous organic compounds formed as a result of direct or indirect biological action (classified as alkanes, cycloalkanes or naphthenes, aromatics, asphalts, and combinations of these) are components of crude oils. Asphaltic compounds in petroleum contain oxygen (O), sulfur (S), and nitrogen (N), and hence are not true hydrocarbons. The principal complexes of these compounds are asphaltenes and resins, both of which contain a large proportion of aromatics. For example, Pennsylvania crude oil contains nearly 100% hydrocarbons (i.e., 85% carbon and 13.5% hydrogen) with the bulk as *n*-alkanes, whereas midcontinent and gulf oils range from 90 to 95% hydrocarbons and California and Mexico oils (including Tia Juana crude) are composed of approximately 50% hydrocarbons and 50% asphaltics including heavy distillate fractions. South Louisiana crude oil contains approximately 30% gasoline, 10% kerosene, 15% light distillate oil, 25% heavy distillate oil, and 20% asphaltic residuum. This crude oil is of low viscosity and contains natural surfactants which promote spreading over water. In contrast, Venezuela crude oil, a high viscosity, low surfactant oil, consists of approximately 10% gasoline, 5% kerosene, 20% light and 30% heavy distillate oils, and 35% asphaltic residuum. Mississippi crude oil has over 50% asphaltic residuum, and bunker C or No. 6 fuel oils, which constitute a major portion of transported refinery products, are the heaviest distillate fractions of petroleum (20, 25, 30, 44).

When oil spills occur, the paramount consideration is the rapid removal of the oil. Since most mishaps have occurred on large bodies of water, relatively little consideration has been given to terrestrial spills, yet numerous petroleum transport systems do involve terrestrial transfer. The trans-Alaskan pipeline will traverse more than 800 miles of ecologically sensitive terrain. In such an environment, physical and mechanical clean-up procedures following an oil spill could cause more damage than the oil itself (25). Disasters with far-reaching and deleterious environmental effects, such as the tanker *Torrey Canyon*,

Santa Barbara channel, and Gulf of Mexico spills are most likely to occur in coastal and estuarine areas (12). The coastal marshes of south Louisiana are adjacent to offshore oil fields; the effects of a major oil spill disaster on such a broad, shallow, salt-marsh estuary are largely speculative since studied oil spills have occurred mostly in coastal areas characterized by cliffs and pocket beaches (37). The need to ship large volumes of hydrocarbon products by sea from the mideast and South America to the industrial centers of western Europe, Japan, and the United States and the development of offshore oil fields will perpetuate the possibility of oil-spill disasters for as long as the world's oil reserves last.

The removal of oil from a body of water involves the collection and physical extraction of gross quantities of the spilled oil (14, 31, 39). However, a substantial amount of oil remains after mechanical or chemical techniques are used. Chemical dispersants in extremely large amounts have been employed at most major oil-spill sites. The toxicity of these chemical dispersants has been a major concern, as has the toxicity of the dispersed oil itself (17, 22, 41).

The ultimate clean-up of an oil spill requires biological oxidative activity (4, 7, 10, 21). When oil is left on the sea or in marshes, harbors, rivers, or lakes, stimulation of the rate of normal biodegradation may reduce damage to the environment. Microbial seeding, enriching oil spills with nutrients, or both, may be used since unaided biodegradation does not provide effective relief against massive disaster (4, 7, 10, 28). No single microorganism or combination of microorganisms has completely degraded any crude oil. Microorganisms with enzymatic properties for rapid attack on a broad spectrum of hydrocarbons may be uncommon.

Most research on the microbial degradation of oily pollutants has centered on bacteria or mixtures of unidentified microorganisms (47). Fungi, due to their greater osmotic flexibility and enzymatic capacities, may prove more practical. Ahearn et al. (7) reported that yeasts in oil-bearing regions frequently utilized hydrocarbons as substrates for growth, suggesting in-situ oil biodegradation. Oil enrichment of estuarine and marine environments was shown to initiate increased densities of certain yeasts (4). Further, yeasts from aquatic sites chronically polluted with oil demonstrated higher rates of degradation and emulsification of crude oil than did some common marine-occurring yeasts (such as isolates of *Rhodotorula*, *Debaryomyces*, and members of the *Candida parapsilosis* complex) from apparently oil-free habitats.

Although numerous yeasts demonstrably utilize hydrocarbons, at least to some degree, strains of *Candida tropicalis* and *C. lipolytica* are particularly active (20, 29, 33, 38). In a series of reports (4, 7, 34), isolates of *C. tropicalis* and *C. lipolytica* were found to grow readily on Louisiana crude oil and a variety of its refinery products. Seeding of yeasts to facilitate biodegradation was suggested by Ahearn et al. (7). Definitive studies of the rates of oil degradation by yeasts or of the effects of microbial seeding on the ecology of an oil-

spill site have not been undertaken.

This study examines the ability of selected fungi, particularly strains generally identified with the genus *Candida*, to grow and survive in oil-enriched fresh and marine waters was investigated.

MATERIALS AND METHODS

CULTURES

Fungi were obtained from the culture collection of the Department of Biology, Georgia State University, from the Department of Food Science, Louisiana State University, Baton Rouge, Louisiana, and by direct isolation from sites polluted with crude oil or hydrocarbon distillates. The cultures included isolates from oil-polluted marine and fresh waters and contaminated aircraft fuel systems (Table 1).

TABLE 1. Sources of Fungi

Organism	Source
Candida lipolytica 37-1	Frankfurter (6)
Candida subtropicalis AJ4476	Air (38)
Candida subtropicalis R42	Asphalt refinery (43)
Candida tropicalis NB2	Asphalt refinery (43)
Candida tropicalis W12B	Asphalt refinery (43)
Candida tropicalis 231	Sputum
Candida tropicalis 6418	Skin
Cladosporium resinae SA300	Jet fuel
Cladosporium resinae SA8	Jet fuel
Trichosporon fermentans SA100	Jet fuel
Candida parapsilosis GM181	Oil slick, Gulf of Mexico (35)
Trichosporon cutaneum GM180	Oil slick, Gulf of Mexico (35)
Rhodosporidium toruloides GM183	Oil slick, Gulf of Mexico (35)
Itersonilia sp. JK29	Eye cosmetic (46)
Pichia ohmeri LSU215	Barataria Bay, marsh (35)
Pichia spartinae FST119	Barataria Bay, marsh (35)
Pichia saitoi MENA	Barataria Bay, marsh (35)
Kluyveromyces drosophilarum FST125	Barataria Bay, marsh (35)
Kluyveromyces dobzhanskii N-1	Barataria Bay, oiled plots
Pichia spartinae N-18	Barataria Bay, oiled plots

Direct isolations were made by streaking oily materials onto selected media. These included yeast nitrogen base (YNB, Difco) agar with 0.5 g/l chloramphenicol; Mycosel (BBL) and Mycobiotic (Difco) agars which contain 0.4 g/l cycloheximide and 0.05 g/l chloramphenicol; casein agar

(6) prepared with and without 0.5 g/l chloramphenicol and 0.4 g/l cycloheximide; Mycological (Difco) agar acidified to pH 4.5 with lactic acid; and diamalt agar (5). Media were prepared in both distilled water and seawater.

HYDROCARBON ASSIMILATION

Oil assimilation capacity was assessed by visual determination of fungal growth in 4.8 ml YNB medium (45) supplemented either with approximately 0.2 ml of Louisiana crude oil (a slight residue remained on the inside surface of the pipettes) or with the weight of the more viscous oils (Tia Juana, Venezuela, or Mississippi) equal to the weight of Louisiana crude used. Cells for inoculum were grown in YNB broth with 0.01% glucose for 24 to 48 hr to accomplish carbohydrate depletion. Of this growth, 0.05 ml was used to inoculate each hydrocarbon utilization test. All cultures were incubated at 22 to 26 C on a roller drum set at an angle of 80° and a speed of 40 to 50 rpm. The hydrocarbons were not sterilized; uninoculated controls were incubated to assess any possible contamination. Visual determination of growth was recorded using a 0-3 scale; zero indicated the absence of growth and three indicated maximal growth as compared to a glucose control. The cultures were examined microscopically at various stages during the incubation period.

OXYGEN CONSUMPTION

Oxygen consumption was determined by a modification of the technique of Tool (42) employing a Hach manometric biochemical oxygen demand (BOD) apparatus, Model 2173. Inocula for the respiration studies contained 4×10^7 to 6×10^7 cells from 24-hr YNB broth cultures with 0.5% glucose. Respiration bottles contained approximately 1.0% hydrocarbon (v/v) and 0.01% yeast extract (Difco) in distilled water or seawater. Net oxygen consumption was determined after 72 hr at 6, 20, and 30 C. Although usual BOD procedures require no mixing of bottle contents during the incubation period (8), the procedure used required constant agitation with stirring initiated before the addition of hydrocarbon. Upon completion of the experiment, oily samples from the respiration bottles were streaked on isolation agars to check for culture purity. Control respiration bottles contained either hydrocarbon and yeast extract, inoculum and yeast extract, hydrocarbon only, inoculum only, or yeast extract only. Net oxygen consumption (mg/l) with 1.0% (v/v) crude oil as substrate was determined by subtracting both endogenous respiration and oxygen consumed in auto-oxidation.

EMULSIFICATION OF CRUDE OILS

Tests were conducted to compare the emulsification of Mississippi and Louisiana crude oils by the yeasts *C. subtropicalis* R42 and *C. lipolytica* 37-1. Live cells, dead cells (autoclaved), and lyophilized cells in concentrations of 1×10^{10}, 1×10^{12}, 1×10^{13}, 1×10^{14}, and

1×10^{15} were added to test tubes (18 mm) containing 9.9 ml of sterile tap water and 0.1 ml of crude oil. The various yeast-oil mixtures and control tubes were agitated at 44 rpm on a roller drum at 25 C for one month. Visual observations were made weekly to record the degree of oil emulsification. All tests were run in triplicate. Samples were taken from tubes which displayed the best emulsification of oil after a period of one month. The diameters of 25 oil droplets from each tube were measured microscopically. Additional observations were made on aquaria separately enriched with the two crude oils, with and without the same two yeasts. Adhesiveness of the oil to the glass walls and to wooden paddles was noted as was the visible emulsification of the oil in the various aquaria. The degrees of emulsification and adhesiveness were compared with results from aquaria containing a commercial dispersant, Polycomplex A-11 (Guardian Chemical Corp., Hauppauge, New York).

SURVIVAL OF SEED CULTURES IN FRESH WATERS

Selected fungi were examined for their ability to survive and multiply in simulated oil spills. Water from an asphalt refinery holding pond (heavily oil polluted; for description of this site see Turner and Ahearn [43]) was used as diluent for approximately 4% (v/v) Louisiana crude oil or bunker C fuel oil. Buckets, containing a total volume of five liters, were employed as testing containers. Each was equipped with a cover to prevent addition of water by precipitation. One bucket in each test series was enriched with 25 g of $(NH_4)_2SO_4$. The test buckets were placed in the open in the vicinity of Atlanta, Georgia, between December 1970 and May 1971. A mixture of nine fungi was used as inocula for the containers. The cell crop for each organism, after 48 hr growth in five ml of YNB broth with 0.5% glucose, was obtained; the inoculation mixture was prepared by pooling the resultant broth cultures. Samples from test and control buckets were taken with sterile dacron swabs at 3 to 5 week intervals over a period of five months. Isolation agars were surface streaked with a swab and incubated at room temperature. Developing fungi were isolated and identified as previously described (4).

In a separate series of field tests, water from a non-oil polluted lake was enriched with approximately 4% (v/v) Louisiana crude oil; all tests employed a final volume of five liters. The inoculum consisted of a mixture of six yeasts and one filamentous fungus (1.0 ml each from 24 hr cultures grown on YNB broth with 0.5% glucose). Samples from test and control buckets were taken over a period of twelve months (August 1971 to August 1972) and the fungi were isolated and identified.

ESTUARINE STUDIES

Water, sediment, and vegetation (*Spartina alterniflora*) samples were taken from the northwestern region of Barataria Bay, Louisiana, at intervals between August 1971 and July 1973, and examined for their fungal

flora. Detailed descriptions of the collection area and the isolation and identification procedures have been published (35, 36). Maintenance of the seed plots in the estuary was facilitated by the use of 50-gal (189-liter) drums with both ends removed and with lateral holes below the water line. These barrels were positioned in the estuarine waters and inoculated with a mixture of either growing cells or lyophilized cells of six fungi, including five yeast and one filamentous isolate. Louisiana crude oil was added to all barrels at 3 to 5 week intervals over an eight-month test period. The mixed inoculum of growing cells consisted of a total of 30 ml from 24-hr broth cultures (2.0% glucose, 1.0% peptone, and 0.5% yeast extract broth prepared with seawater). The cell crops obtained from 48-hr cultures of each of the same six fungi (grown on medium of the above formulation with 2.0% agar) were lyophilized and used as inocula for additional field tests.

In subsequent seed culture tests in the marsh, only *Candida lipolytica* 37-1 and *C. subtropicalis* R42 were used for inoculation of sediment plots. One hundred ml of Louisiana crude oil was applied to the plots prior to inoculation and then periodically over an eleven-month test period. Duplicate samples from the estuarine barrels were filtered through cellulose-ester membranes (Millipore Filter Corp.) of 0.45 µm porosity, and the membranes were implanted aseptically onto distilled and seawater isolation media. Sediment and vegetation samples from the oiled plots were taken with sterile utensils and plated directly onto isolation media. Occasional samples were kept on ice for 5 to 10 hr prior to culturing. Part of each sediment sample was diluted with sterile seawater, vigorously agitated, and the supernatant was filtered through cellulose-ester membranes; the membranes were then aseptically implanted onto isolation media. Control samples were taken from the natural bay waters, sediments, flora, and from oil-seeded, uninoculated barrels and marshland plots. Representative yeasts and filamentous fungi were selected from the isolation plates on the basis of colony morphology. Identification procedures for yeasts followed the methods of Wickerham (45) and Lodder (32).

RESULTS

ISOLATION PROCEDURES

Hydrocarbonoclastic fungi grew readily when oily residues from the environment were streaked on YNB agar without carbon enrichment. Yeast and fungal colonies were selected from the isolation plates, usually after incubation for 72 or more hr. Isolation of yeasts was facilitated when the antibiotics chloramphenicol and cycloheximide were added to the medium.

The latter compound, a di-ketone, is inhibitory to numerous saprophytic fungi, but two of the most active hydrocarbonoclastic yeasts (*Candida lipolytica* 37-1 and *C. subtropicalis* R42 and AJ4476) were resistant to 0.4 g/l cycloheximide. Most strains of *C. tropicalis*

(including clinical isolates) which hydrolyzed and utilized starch and gave fair to good growth on crude oil, were also resistant to cycloheximide. Other starch-utilizing strains of *C. tropicalis* (e.g., 6418, Table 1) were susceptible to 0.4 g/l cycloheximide and gave latent growth on crude oil. Certain yeasts (when isolated from oil-soaked plots) proved resistant to cycloheximide, yet representative clinical isolates of the same species were susceptible to this antibiotic (e.g., species of *Cryptococcus* [1]). *Candida lipolytica* 37-1 produced significant amounts of extracellular proteinase as indicated by the development of a clear zone in casein agar (6). This property of *C. lipolytica*, along with the resistance of both *C. lipolytica* and *C. subtropicalis* to cycloheximide and their inability to hydrolyze starch, permitted a ready distinction of these yeasts.

OIL UTILIZATION AND EMULSIFICATION

Most yeast isolates grew within three days with Louisiana crude oil as the sole source of carbon (Table 2). The heaviest growth on Louisiana crude oil after three days incubation was by strains of *C. lipolytica*, *C. subtropicalis*, *C. tropicalis*, and *Trichosporon fermentans*. In contrast to most yeasts tested, both *C. subtropicalis* R42 and AJ4476 and *C. lipolytica* 37-1 produced weak growth on Tia Juana crude oil by ten days. Generally, growth of yeasts with either Tia Juana, Venezuela, or Mississippi crude oil as substrate was minimal and evident only after twenty days incubation. *Candida subtropicalis* R42 and *C. lipolytica* 37-1, in particular, emulsified the crude oils; both the cell-free, spent-culture broths and whole cells showed emulsifying properties.

The mean diameters of oil droplets of both Louisiana and Mississippi crude oils generally decreased with an increased concentration of yeast cells (Table 3). In general, live yeast cells enhanced the formation of smaller oil droplets than did either dead or lyophilized cells. The adhesion of crude oil to glass test tubes or to wood paddles decreased immediately upon addition of live, dead, or lyophilized yeast cells. This dispersive effect was enhanced by increased concentrations of cells. Emulsification of the crude oil in test tubes inoculated with live yeasts, as determined by weekly visual observations, increased with time and yeast growth over a one-month period. This same effect was noted in the aquaria which were enriched with either of the two crude oils and live yeasts. Cells of *C. lipolytica* 37-1 were more effective dispersants of Louisiana crude oil, whereas cells of *C. subtropicalis* R42 gave visual evidence of greater emulsification of the more viscous Mississippi crude oil. In general, Mississippi crude was not as dispersed as the Louisiana crude, although the average size of measurable oil droplets of Mississippi crude oil was smaller than those of Louisiana crude. Many more large, hardened globules above one millimeter in diameter were present in the tests involving Mississippi crude than in those with Louisiana crude.

TABLE 2. Relative Growths of Fungi with Louisiana Crude Oil as a Carbon Source

Organism	Growth[a]	
	3 days	10 days
Candida lipolytica 37-1	3	3
Candida subtropicalis AJ4476	2	3
Candida subtropicalis R42	2	3
Candida tropicalis NB2	2	3
Candida tropicalis W12B	2	3
Candida tropicalis 231	1	3
Candida tropicalis 6418	1	3
Cladosporium resinae SA300	0	1
Cladosporium resinae SA8	0	1
Trichosporon fermentans SA100	3	3
Candida parapsilosis GM181	1	3
Trichosporon cutaneum GM180	1	2
Rhodosporidium toruloides GM183	1	2
Itersonilia sp. JK29	1	2
Pichia ohmeri LSU215	1	3
Pichia spartinae FST119	0	0
Pichia saitoi MENA	0	0
Kluyveromyces drosophilarum FST125	0	0
Kluyveromyces dobzhanskii N-1	0	0
Pichia spartinae N-18	0	3[b]

[a]Growth on YNB medium with 4% (v/v) Louisiana crude oil. Visual determination of growth: 0 negligible, 3 maximal.

[b]Growth after 20 days incubation; cells aberrant.

The mixture of either crude oil with either yeast was more effective in reducing the adhesiveness of the oil to glass than was the mixture of Polycomplex A-11 dispersant with either oil. This effect was noted in both test tubes and aquaria. Microscopic examination of agitated cultures showed that these yeasts coated the subsurface oil globules with budding cells, whereas in surface slicks in non-agitated systems, mats of hyphae were formed within and upon the oil layers. Similar growth results were obtained from media prepared with either fresh water or seawater. *Rhodosporidium toruloides* GM183 and numerous other red yeasts (species of *Rhodosporidium* and *Rhodotorula*) isolated from oil-polluted areas utilized components of crude oil for growth and energy, but never with amounts of growth or emulsification comparable to those of the species of *Candida*. *Pichia spartinae* FST119, *P. saitoi* MENA, and *Kluyveromyces drosophilarum* FST125 from relatively oil-free estuarine marsh sites failed to grow with oil as a sole carbon source even after ten days incubation.

TABLE 3. Average Diameter of Crude Oil Droplets Emulsified by Yeasts[a]

Cell type	Louisiana Crude				Mississippi Crude			
	C. tropicalis		*C. lipolytica*		*C. tropicalis*		*C. lipolytica*	
	Diam[b]	Range[c]	Diam	Range	Diam	Range	Diam	Range
Autoclaved	101	20-580	82	20-385	63	10-305	55	15-230
Lyophilized	80	15-405	58	10-250	44	10-200	51	10-530
Live	83	15-450	51	10-180	54	10-515	94	10-335
Control (no yeast)	165	50-925			574	20-2870		

[a]Measurements in micrometers by ocular micrometer; sample from suspended material after agitation for 25 days.

[b]Mean diameter, 25 globules; globules larger than 1 μm not considered.

[c]Size range of globules; globules larger than 1 μm not considered. Adapted from Cook et al. (19).

SURVIVAL OF SEED CULTURES IN FRESH WATERS

A mixture of eight fungi (*C. tropicalis* NB2 and W12B, *C. lipolytica* 37-1, *C. parapsilosis* GM181, *Trichosporon fermentans* SA100, *Cladosporium resinae* SA300, *Trichosporon cutaneum* GM180, *Rhodosporidium toruloides* GM183, and *Itersonilia* sp. JK29) was introduced into fresh water obtained from an asphalt refinery drainage system. The water was enriched with Louisiana crude or bunker C oil. The most noticeable effects of the fungi on the oiled water were partial disruption of the surface slick, development of a more evident oil-in-water emulsion, and the formation of matted, irregular hyphal layers at the oil-water interphase. Such phenomena were much less evident in the control buckets. Periodic samplings of the test systems followed by isolation and identification of yeasts over a five-month period demonstrated a gradual decrease in certain populations, the disappearance of a few species, and the establishment of wild fungi in both test and control systems (Table 4). Approximately the same numbers of fungi were obtained from test buckets with and without enrichment with 25 g of $(NH_4)_2SO_4$. Less microbial activity was evident in the bunker C fuel oil systems than in the Louisiana crude. Species of *Cladosporium* which gave negligible evidence of growth on crude oil in pure culture systems were the predominant isolates from the bunker C field tests (Table 4). Bacteria were present in all test systems, particularly in those enriched with bunker C fuel oil. Neither oil was completely degraded during the five-month test period.

A second series of seed-culture tests involved fresh water from a non-oil polluted lake; the water was enriched with 4% (v/v) Louisiana crude oil. Containers were inoculated with a mixture of seven fungi

TABLE 4. Predominant Fungi Recovered from Asphalt Refinery Lake Waters Enriched with 4% (v/v) Hydrocarbons

	Louisiana crude oil						Bunker C fuel oil[a]			
	Inoculated			Uninoculated			Inoculated		Uninoculated	
	1 mo.	3 mo.	5 mo.	1 mo.	3 mo.	5 mo.	1 mo.	3 mo.	1 mo.	3 mo.
Yeast:										
Candida tropicalis[b]	25[c]	30	40	/[d]	/	5	10	5	/	/
Candida lipolytica[b]	25	25	20	/	/	/	10	5	/	/
Candida parapsilosis[b]	/	/	10	/	/	/	/	/	/	/
Trichosporon fermentans[b]	15	20	10	/	/	/	75	15	/	/
Red yeast	25	20	13	50	25	25	5	5	50	50
Filamentous fungi:										
Cladosporium sp.[b]	5	/	7	/	/	/	/	50	/	/
Cephalosporium sp.	/	5	5	5	25	25	/	5	5	5
Fusarium sp.	/	/	/	5	25	25	/	5	5	5
Penicillium sp.	/	/	/	5	5	10	/	5	5	5
Alternaria sp.	/	/	/	5	15	10	/	/	/	5
Aspergillus sp.	/	/	/	5	5	/	/	/	/	5
Trichoderma viride	/	/	/	/	/	/	/	5	/	5

[a]Bacterial growth predominant in all bunker C buckets.

[b]Organisms seeded into inoculated buckets.

[c]Percent of total fungal isolates.

[d]/ indicates not isolated.

(*Cladosporium resinae* SA300, *Candida parapsilosis* GM181, *Pichia ohmeri* LSU215, *C. tropicalis* NB2, *C. subtropicalis* R42, *Trichosporon fermentans* SA100, and *C. lipolytica* 37-1). Samples from both test and control systems were taken periodically for twelve months. Within three months, the integrity of the initial oil layer (approximately one centimeter) in each inoculated bucket was disrupted, and there was noticeable evaporation of water. At one year, a film of crude oil was no longer present in the inoculated buckets; instead a thick, spongy pellicle covered the water surface; the pellicle had a black, hardened, asphalt-like upper surface and a brown, spongy underside with much stringy mycelial growth. Thick hyphal strands extended down into the turbid water; many strands sank to the bottom of the containers. Only about 500 ml of water remained. The oil film in the control container was reasonably unaltered, and the water volume remaining was approximately four liters at the end of one year. The uninoculated control (water from the non-oil polluted lake) showed little evidence of microbial activity. Of the seven species of fungi introduced into the test systems, four species (*Trichosporon fermentans*, *Cladosporium resinae*, *Candida parapsilosis*, and *Pichia ohmeri*) were never recovered. *Candida lipolytica* was recovered at the end of the first three weeks but not thereafter. *Candida subtropicalis* was obtained on each sampling throughout the year. During this time both control and inoculated buckets were populated with numerous fungi, mainly species of *Alternaria*, *Cephalosporium*, *Fusarium*, and *Penicillium*.

SURVIVAL OF SEED CULTURES IN ESTUARINE WATERS

Prior to the introduction of the seed cultures into estuarine waters species of *Pichia*, *Kluyveromyces*, *Rhodotorula*, and *Cryptococcus* were found to predominate at the test site. No isolates of these species produced strong growth on hydrocarbons before exposure of the marsh site to oil. A mixture of six hydrocarbonoclastic fungi (*Trichosporon fermentans* SA100, *Cladosporium resinae* SA300, *Candida tropicalis* NB2, *C. subtropicalis* R42, *C. lipolytica* 37-1, and *C. parapsilosis* GM181) was introduced into estuarine waters in Barataria Bay, Louisiana. Colony-forming units (cfu) of *C. subtropicalis* and *C. lipolytica* constituted approximately 95% of all microorganisms recovered from water samples taken 24 hr after inoculation. A few red yeasts were also evident. No *Trichosporon* or *Cladosporium* species were recovered.

After the first sampling, high tides in the fall of the year completely inundated the test barrels; both the seeded crude oil and the top stratum of water were washed away. Samples for fungal identification were taken and Louisiana crude oil again was applied to the plots, but the plots were not reinoculated with yeasts. Analysis of the samples indicated that the indigenous fungi had returned and the seeded *Candida* species were lost. Of the fungi isolated, approximately 40% of the cfu were species of *Pichia*, 20% *Kluyveromyces*, 20% *Cryptococcus*, 10% red yeasts, and 10% filamentous fungi. *Cladosporium* species were rarely isolated; those isolates examined were morphologically distinct from the "kerosene fungus" *Cladosporium resinae* (40). Almost eight months after the original inoculation, high spring tides washed some of the barrels out to sea. Essentially the same isolations of fungi as those reported earlier were made. Additions of oil during the interim period had not encouraged evident growth of organisms other than the indigenous fungi.

SURVIVAL OF SEED CULTURES IN THE MARSH

The yeasts *Candida lipolytica* 37-1 and *C. subtropicalis* R42 were introduced into marshland sediments; the test plots were enriched with Louisiana crude oil both prior to inoculation of the yeasts and periodically thereafter.

Samples from the plots were taken before inoculation and after two months, three months, seven months, and eleven months (Table 5). Although the yeasts *C. subtropicalis* R42 and *C. lipolytica* 37-1 were not found in Barataria Bay prior to their inoculation as seed cultures, they persisted for over seven months in oiled plots in the marsh. *Candida subtropicalis* was recovered in greater densities from all samples of the oil-soaked plots. Neither yeast was isolated from adjacent non-oiled sites throughout the entire study. The seed yeasts survived the seasons from late fall to late spring or early summer. Representatives of *Pichia spartinae* with the ability to utilize and emulsify crude oil

TABLE 5. Fungi Isolated from Oiled Sediments, Barataria Bay, Louisiana

	Prior to inoculation	Time after inoculation of seed cultures											
		2 months			3 months			7 months			11 months		
	Sites 1 & 2	Site 1	Site 2	Control[a]	Site 1	Site 2	Control	Site 1	Site 2	Control	Site 1	Site 2	Control
Seed Cultures:													
Candida subtropicalis R42	0[b]	+	+	0[c]	+	+	0	+	+	0	0	0	0
C. lipolytica 37-1	0	+	+	0	+	+	0	+	+	0	0	0	0
Indigenous Fungi:													
Yeast-like:													
Rhodotorula-Rhodosporidium sp.	+	+	+	+	+	+	+	+	+	+	+	+	+
Cryptococcus sp. (*C. albidus* and *laurentii*)	+	+	+	+	+	+	+	+	+	+	+	+	+
Kluyveromyces sp. (*K. drosophilarum* and *dobzhanskii*)	+	+	+	+	+	+	+	+	+	+	+	+	+
Pichia sp. (*P. spartinae*, *saitoi*, and *ohmeri*)	+	+	+	+	+	+	+	+	+	+	+	+	+
Trichosporon sp. (mostly *T. cutaneum*)	+	+	0	+	+	0	+	+	+	+	0	+	+
Aureobasidium sp.	+	0	0	+	0	0	+	0	0	+	0	0	+
Filamentous:													
Cladosporium sp.	+	0	0	+	+	+	+	0	0	+	+	0	+
Penicillium sp.	+	0	+	+	+	+	+	0	0	+	0	+	+
Cephalosporium sp.	+	+	+	+	+	+	+	+	+	+	+	+	+
Fusarium sp.	+	+	+	+	+	+	+	+	+	+	+	+	+
Alternaria sp.	+	+	0	0	0	0	+	0	0	+	0	0	0
Trichoderma viride	0	0	+	+	0	0	0	0	0	+	0	0	+
Aspergillus sp.	0	0	0	0	0	0	0	0	0	0	+	+	0

[a]Control plots, periodically oiled, no inoculum.

[b]0 indicates not isolated, + present.

[c]Seed cultures introduced in non-oiled sediments were not recovered after 72 hr.

were isolated after the plots were subjected to oil for at least several months. Microscopic examination of the growth on oil revealed that the yeast produced short chains of aberrant, pseudo-hyphal cells interspersed with numerous chlamydospores. Of the major species indigenous to the area, no other hydrocarbonoclastic isolates were found. Filamentous fungi with weak hydrocarbonoclastic properties, including species of *Trichosporon* (mainly *T. cutaneum*), *Aureobasidium*, *Penicillium*, and *Cephalosporium* were obtained throughout the study.

OIL OXIDATION BY SELECTED SEED CULTURES

Oxygen consumption by *Candida lipolytica* 37-1 at 20 C with various crude oils as substrate was compared in both distilled water and filtered seawater (Table 6). Significant oxygen consumption within a 72-hr period was obtained only with Louisiana or Tia Juana crude oil as substrate. No essential difference in results obtained with distilled water or seawater was noted.

TABLE 6. Oxygen Consumption by *Candida lipolytica* 37-1 after 72 Hours at 20 C with Crude Oil as Substrate[a]

Crude oil	Distilled water	Filtered seawater
Louisiana	352[b]	355
Tia Juana	175	215
Venezuela	10	10
Mississippi	10	10

[a]1.0% crude oil with 0.01% yeast extract added.

[b]Mg oxygen/l, average of 3 repeat tests.

The oxygen consumption at 72 hr with 1.0% Louisiana crude oil as substrate for *C. lipolytica* 37-1 and *C. subtropicalis* R42 was determined at 6, 20, and 30 C (Table 7). Both yeasts had scant oxygen uptake at 6 and highest activity at 20 C. The maximum growth temperature of *C. subtropicalis* R42 on Mycological agar was about 42 C; this yeast did not grow at 45 C. *Candida lipolytica* 37-1, unlike most isolates of this species (32) grew readily at 30 C and produced only slight growth at 37 C.

TABLE 7. Oxygen Consumption by *Candida lipolytica* 37-1 and *Candida subtropicalis* R42 after 72 Hours with Louisiana Crude Oil as Substrate[a]

Temperature C	37-1		R42		Uninoculated (auto-oxidation)
	Oil	Control[b]	Oil	Control[b]	
6	40[c]	0	25	0	0
20	352	40	313	45	35
30	315	30	270	25	30

[a]1.0% crude oil with 0.01% yeast extract added, distilled water.

[b]In 0.01% yeast extract, distilled water, no crude oil.

[c]Mg oxygen/l, average of 3 repeat tests; ±25 mg O_2/l at 20 and 30 C, ±5 at 6 C.

GENERAL ECOLOGICAL OBSERVATIONS

No adverse ecological conditions were observed which could be attributed to addition of yeasts to water containers or sediment plots. In microscopic examinations of fresh water material, a variety of protozoa was observed. These included members of the genera *Phacus*, *Stylonychia*, *Paramecium*, and *Amoeba*, some of which were seen feeding on yeast cells.

In marine plots, the gastropod *Nerita*, a grazing herbivore, apparently infiltrated oil sites. In the sediments, some nematodes and protozoa were observed. These populations in the oiled plots, seeded with yeasts, appeared to be equivalent to or higher than those in control plots.

DISCUSSION

The indigenous yeast flora of pristine fresh water and estuarine environments demonstrated limited capacities to utilize crude oils as contrasted with yeasts from sites enriched with petroleum. Only after about a year of exposure to oil did a few representatives of the indigenous flora show hydrocarbonoclastic activity. Hydrocarbonoclastic fungi introduced into oil-soaked habitats did not all survive. Of the fungi used, isolates of *C. subtropicalis* and *C. lipolytica* persisted in both fresh water and estuarine environments enriched with oil for varying periods of time without apparent adverse effects on the ecology of the study sites. Moreover, these yeasts seemed to be localized at the oiled plots. *Candida subtropicalis* persisted the longest at both fresh water and estuarine sites.

Ahearn (3) reported that *C. tropicalis* was found generally in polluted waters which had a BOD in excess of 2 mg/l. This report made no reference to *C. subtropicalis*. Nakase et al. (38) differentiated *C. tropicalis* from *C. subtropicalis* using properties such as starch utilization, hydrocarbon assimilation, maximum growth temperature, DNA base composition, and antigenic distinctions. Culture R42 and the type strain of *C. subtropicalis* (AJ4476) both were unable to utilize starch, did not grow well at temperatures over 42 C, and gave rapid growth with hydrocarbons as sole carbon sources. In addition, both cultures were resistant to 0.4 g/l cycloheximide. The natural surfactant of *C. subtropicalis* R42 more effectively dispersed Mississippi crude oil (which contains over 50% asphaltics) than did the yeast *C. lipolytica*. Guire et al. (23) identified the peptidolipid surfactant of *C. petrophilum* ATCC20226 (synonym *C. lipolytica*) and reported it relatively nontoxic for the water flea *Daphnia magna*, stable to high temperatures, functional over a wide pH range, and susceptible to hydrolytic enzymes. Analysis of the surfactant from *C. subtropicalis* has not been reported.

The most obvious emulsification effect noted was the decrease in adhesiveness of both crude oils to glass walls (test tube and aquaria) and wood paddles when cells of either yeast were added. Lyophilized and live yeast cells had similar emulsification effects on both Louisiana and Mississippi crude oils. The differences in degrees of emulsification noted in comparison tests of the two oils could be attributed in part to their content of short chain hydrocarbons and their viscosities. Generally, the greater the number of yeast cells present, the smaller the average size of emulsified oil droplets. The natural surfactant material of both yeasts (*C. subtropicalis* R42 and *C. lipolytica* 37-1) had only negligible toxic effects on guppies (19). These same yeast surfactants when added to oil films in beakers acted as oil herders, concentrating the oil in the center of each beaker. Microscopic observations indicated that an increase in the number of oil droplets present as well as a decrease in droplet diameter accompanied the decrease in adhesiveness as cell numbers increased. Such an effect should have far-reaching environmental significance when the removal of pollutant oils from an area is considered. In the natural biodegradation of oils, the amount of surface area available to form an oil-water interface may be rate limiting. An increase in surface area accomplished by the presence of very small droplets should enhance microbial degradation of the oils.

Candida subtropicalis R42 was able to degrade a broad range of alkanes and alkenes, grew and multiplied in both fresh water and seawater, remained stable under adverse conditions, emulsified oils, and most importantly, once established in oil-seeded marine plots did not spread into the environment. No literature reports on the pathogenicity of *C. subtropicalis* for man or animals were found. All of these properties are important for strains used as seed cultures to facilitate the biodegradation of pollutant oils. *Candida subtropicalis* possesses characteristics which warrant further research into its practical applications in combatting oil pollution.

Numerous technical and practical considerations suggest that hydrocarbonoclastic seed cultures will have restricted applications. Ahearn (2) stated that further research was needed as only cursory information was available on the immediate and long-term effects of the microbial seeding of oil spills. Bartha and Atlas (11) indicated that nitrogen and phosphorus are limiting factors for oil degradation in seawater. In a subsequent report, these researchers suggested that an oleophilic fertilizer (e.g., a combination of octylphosphate and paraffinized urea) could be employed to increase the rate of natural degradation (10). Actual field tests of this process have not been reported.

In compiling the weathering history of two light paraffinic crude oils which stranded on Martha's Vineyard, Massachusetts, and on Bermuda, Blumer et al. (13) noted the great persistence of spilled oil, and concluded that its half life must be measured in years. Our studies suggest that fertilization techniques for open environments may not be readily effective in regions normally free of oil and thus lacking an indigenous hydrocarbonoclastic flora. Jobson et al. (26) and Anderes (9) also found that non-oil polluted sites lacked significant populations of organisms capable of crude oil utilization. Moreover, the fertilization of an open aquatic system could result in eutrophication with the development of unwanted species. Therefore, seed systems may find their primary application in refinery waste treatment systems or in facilitating the removal of sludge oils from shipboard installations. In such systems, growth conditions could be partially controlled, and if necessary, nutrient enrichment or natural surfactants could be used. Although complete utilization of crude oil has not been achieved by any culture system, a microbial seed system which emulsified as well as utilized part of the crude oil would still be of practical value in removing oils from contained situations.

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MICROBIOLOGICAL ASPECTS OF OIL INTRUSION IN SOUTHEASTERN LOUISIANA

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ABSTRACT

Microbial populations in experimental oiled marsh plots and from the site of an oil spill in the Barataria Bay of Louisiana were monitored for occurrence and density of various physiological types. Results indicate that oil stimulates an increase in hydrocarbonoclastic and proteolytic microorganisms, and a decrease in cellulolytic microorganisms.

Microbiological samples collected along a transect from near-shore waters and sediments to a fresh-water swamp in southeastern Louisiana showed similar increase in hydrocarbonoclasts in areas exposed to oil. A greater diversity of bacterial species was also observed in the oiled regions.

INTRODUCTION

The ability of numerous microorganisms to partially degrade oil under certain simulated environmental conditions is well documented. However, knowledge of the effects of crude oil on microbial processes in the environment is, in the main, restricted to several rather broad observations. As early as 1922, Baldwin (2) noted changes in microbial activity of a corn field after its treatment with petroleum. Such modifications following the introduction of oil included increased numbers of heterotrophs, reduction in nitrate production, decreased diversity of bacterial species, and slightly lowered ammonia production. Kincannon (5) reported alterations in microbial populations of soil treated with crude oil and presented evidence for microbial succession and the predominance of yeasts in several samples. More recently (1973), Cobet and Guard (3) found no noticeable changes in the microbial communities of a beach contaminated by bunker fuel, especially in diversity of bacterial genera with either time or depth in the sand.

Considerations of the possible effects of crude oil intrusion on microbial processes are of particular significance in the coastal wetlands of Louisiana. Implications of such intrusion on the marshland

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microbial ecosystem have been discussed elsewhere (6). Numerous petroleum industries are active in these estuaries which are of primary importance to the fishery resources of the state. The work reported here is concerned with effects of production of crude oil on extant microbial processes in the salt-marsh estuary. Microbial conversion of the primary plant producer (*Spartina alterniflora*) to utilizable substrates is essential to the overall energy flow of the salt-marsh environment.

An ancillary phase of this work was a study of the distribution of hydrocarbonoclastic bacteria over a wide spectrum of habitats including beach zone salt marsh, brackish marsh, fresh marsh and swamp forest of cypress-tupelo gum. The total transect involved a distance of approximately 80 miles.

MATERIALS AND METHODS

Sediment samples were collected at sites as previously described (7). Samples were placed in sterile petri plates and enclosed in sterile Whirl-Pak bags. All materials were then stored in an ice chest for return to the laboratory (within 24 hr), with final processing within 24 hr. Sampling locations were in several regions according to the objectives of each specific phase of the investigation. Primary sampling sites were:

1) Oil retention plots and control marshes, located near the entrance to Airplane Lake (Grand Isle, Louisiana). The oil plots were established in November, 1970, and were treated with 250 ml of oil each month over a nine-month period.
2) Martigan Point, a small island, located NW of Grand Isle in the Barataria Bay region. This site received oil following a break in an oil pipeline in October, 1972. Several areas of this island were ignited and burned as part of the standard cleanup procedures used to remove standing spilled oil.

The second phase of this study involved collection of material for examination along the transect of a proposed pipeline from near-shore marine environments to cypress-tupelo gum fresh-water swamps. Table 1 gives a brief description of the environments of the wetlands transect sampling sites.

For the initial study, standard enumeration techniques were used to document populations and activities of 1) total heterotrophic bacteria; 2) total yeasts; 3) cellulolytic bacteria; 4) cellulolytic fungi, including yeasts and filamentous fungi; 5) proteolytic bacteria; 6) proteolytic fungi; 7) hydrocarbonoclastic bacteria; and 8) hydrocarbonoclastic fungi. For the transect investigation, only total heterotrophic bacteria and hydrocarbonoclastic bacteria and fungi were enumerated. All determinations were of aerobic microorganisms only. Cellulolytic populations were evaluated using a modified MPN-method, with carboxymethyl-cellulose (CMC) incorporated as sole carbon source in the medium. Inoculated tubes were compared with uninoculated controls, and those

containing medium with reduced viscosity were recorded as positive. Hydrocarbonoclastic microorganisms were enumerated using the modified MPN-method described by Gunkel (4).

TABLE 1. Wetland Transect Stations

Station Number	Environment
1	Gulf of Mexico: Offshore from beach (1 m depth) located between Belle Pass and Caminada Pass. A = water sample; B = submerged sediment.
2	Intertidal area of beach Station 1
3	Fourchon area near Bayou Lafourche. Salt marsh system.
4	West end of Fourchon area: *Spartina* marsh.
5	Leeville Oil Field: *Spartina* marsh.
6	Bayou Sevin, north of Leeville: *Spartina* marsh.
7	Point au Chien, Louisiana Wildlife and Fisheries Commission Wildlife Refuge: Brackish marsh dominated by mixed *Spartina alterniflora*, *S. patens*, *Disthchlis spicata*.
8	Bayou Blue north of Bully Camp Oil Field: transitional area between intermediate and fresh marsh: Dominant vegetation, *Sagittaria falcata*.
9	Bayou Boeuf, east of Lake Boeuf.
10	Bayou Citamon, cypress-tupelo swamp on east side of Bayou Lafourche.

Following enumeration, selected organisms were isolated for further physiological studies. Those with cellulolytic or proteolytic ability were tested for inhibition of growth by crude oil using oil-saturated discs (1 cm) placed on plates seeded with test organisms. Measurement of inhibition of enzyme activity was accomplished in an enriched seawater medium containing appropriate substrates, i.e., skim milk or carboxymethyl-cellulose. Growth and activity in media containing 2% Louisiana crude oil was compared to controls without oil to ascertain any reduction in activity of the particular enzyme systems.

RESULTS AND DISCUSSION

ENVIRONMENTAL MONITORING

Table 2 summarizes the densities of proteolytic, cellulolytic, hydrocarbonoclastic bacteria, and total heterotrophic aerobic bacteria.

The total populations of aerobic heterotrophs from all stations in Airplane Lake were comparable. Addition of oil increased concentrations of hydrocarbonoclasts by a factor of 100, slightly increased numbers of proteolytic forms, but resulted in the reduction in the biomass of cellulolytic bacteria. Samples from Martigan Point, collected 4 months after an oil spill, showed similar trends. In both Martigan Point and Airplane Lake, samples of non-oiled sediments showed cellulolytic populations greater than the hydrocarbonoclastic portion of the populations. In sediments exposed to oil, the relationship was reversed, i.e., hydrocarbonoclastic organisms were more numerous than cellulolytic species.

Average heterotrophic and hydrocarbonoclastic populations from six bimonthly samplings of the ten transect sites are given in Table 3. The ratio of hydrocarbonoclasts to heterotrophs is expressed on a percentage basis. The higher ratio obtained at Station 5 (Leeville Oil Field) is indicative of enrichment of hydrocarbonoclasts in an environment subjected to chronic low-level oil intrusion. A ratio of >10 may be used as an indicator of an oil-stressed environment, while pristine habitats regularly have ratios of <10. The Leeville station consistently demonstrated the highest number of different species.

LABORATORY STUDIES

Of 54 isolates studied, 14 (25%) possessed both proteolytic and hydrocarbonoclastic ability. Only 4 (7%) exhibited both cellulolytic and proteolytic activity, while 3 (5%) were cellulolytic and hydrocarbonoclastic. Nine (16%) of the isolates were proteolytic, and 14 (25%) were hydrocarbonoclastic. Five (9%) exhibited none of these capabilities and a similar number utilized all three substrate types. None of the isolates demonstrated cellulolytic ability alone.

None of the yeasts examined possessed proteolytic activity, while only one utilized CMC. This latter organism, a *Trichosporon* sp., was also weakly hydrocarbonoclastic. Hydrocarbon utilization was observed in 16 (75%) of the yeasts studied. The almost complete absence of yeasts with extracellular proteases is not surprising since few yeasts possessing such activities have been noted (1). Only 3 isolates of filamentous fungi (*Epicoccum*, *Fusarium*, and *Alternaria*) used both hydrocarbons and cellulose, whereas none of the isolates possessed hydrocarbonoclastic activity alone.

The higher frequency of bacteria exhibiting both proteolytic and hydrocarbonoclastic activities may explain the increased biomass of proteolytic organisms in areas exposed to oil. Similarly, the lower number

TABLE 2. Populations of Heterotrophic Bacteria in Oiled and Non-Oiled Marsh Sediments, 1973

	Concentration of bacteria (log 10/g wet sediment)					
	Jan	Feb	Mar	May	June	July
Airplane Lake Site						
Control						
total	7.72	7.93	8.36	7.99	8.60	7.60
cell.	4.97	6.34	6.15	5.85	4.36	6.20
prot.	5.78	6.57	6.45	6.59	6.30	6.45
hyc.	5.52	4.78	5.36	4.89	3.90	4.23
Oil Plot-A						
total	7.73	8.11	7.85	7.49	8.11	7.81
cell.	3.89	5.23	5.15	4.25	2.95	5.21
prot.	6.08	6.38	6.30	6.20	6.60	6.78
hyc.	6.41	6.34	3.15	5.89	6.69	6.45
Oil Plot-B						
total	7.84	8.11	8.32	7.96	8.18	7.43
cell.	4.22	5.63	5.49	5.04	2.85	5.20
prot.	6.18	6.69	6.48	6.39	6.81	6.08
hyc.	6.52	7.20	6.66	6.50	7.36	5.60
Martigan Point, Oil Spill Site						
Station No. 1						
total	8.15	8.87	--	9.65	8.30	7.68
cell.	4.98	7.34	--	5.98	4.41	3.85
prot.	6.25	7.00	--	6.58	5.28	6.15
hyc.	6.60	7.73	--	7.04	5.53	6.96
Station No. 2						
total	8.40	9.48	--	9.59	8.48	7.73
cell.	--	7.34	--	5.84	3.08	3.84
prot.	--	6.75	--	6.21	5.08	5.46
hyc.	7.23	7.96	--	6.84	6.15	6.89
Control						
total	--	--	--	7.81	8.70	7.95
cell.	--	--	--	5.45	5.38	4.70
prot.	--	--	--	5.60	5.49	5.40
hyc.	--	--	--	4.84	4.95	4.70

cell. = cellulolytic; prot. = proteolytic;
hyc. = hydrocarbonoclastic

of isolates with both cellulolytic and hydrocarbonoclastic capacities may explain the decrease in the percentage of cellulose utilizers in areas exposed to oil. However, this does not preclude the possible inhibition of cellulolytic microorganisms by oil.

TABLE 3. Total Heterotrophic and Hydrocarbonoclastic Bacteria along Wetland Transect

Station No.	Average No. Total Heterotrophs	Cells/wet wt sample Hydrocarbonoclasts	Ratio of Hydrocarbonoclasts to Total Heterotrophs (Expressed as percent)
1A	1.1×10^4	8.53×10^1	.85
1B	2.9×10^4	9.06×10^2	3.12
2	8.7×10^5	5.80×10^3	.66
3	4.5×10^7	9.48×10^5	2.11
4	7.0×10^7	4.16×10^5	.59
5	2.0×10^7	2.18×10^6	10.9
6	1.6×10^7	6.14×10^5	3.84
7	2.0×10^7	3.19×10^5	1.60
8	2.7×10^7	4.83×10^5	1.79
9	3.2×10^7	4.15×10^5	1.30
10	3.1×10^7	4.94×10^5	1.59

In laboratory studies, none of the proteolytic or cellulolytic organisms studied were inhibited by crude oil on a peptone medium. Moreover, neither inhibition of caseinolysis nor carboxymethylcellulose utilization was evidenced with incorporation of oil into the growth medium.

Although numbers of microorganisms per se are not always absolute indicators of microbial activity, use of population levels as a relative indication of environmental activity appears justified when differences in biomass are large. The reduction of cellulolytic microbial populations in areas exposed to oil suggests that oil affects the overall nutrient turnover in the estuarine-salt marsh. This reduction possibly may be attributed to unavailability of substrate due to physical coating of the substrate particles, since crude oil did not inhibit the carboxymethylcellulose metabolism of selected isolates.

Increased populations of hydrocarbonoclasts observed at Airplane Lake (experimentally-oiled plots), Martigan Point (single spill), and

Leeville oil field (low-level chronic pollution) indicate that similar enrichment processes were operative at all three levels of oil pollution. Increased species diversity, i.e., occurrence of different colonial types, noted at the Leeville oil field suggests that chronic oil pollution may induce a different population than that found after single spill incidents. This is in contrast with the report of decreased species diversity at oil spill sites (2). These differences, in all likelihood, can be attributed to a range of factors, including inherent dissimilarities of the environments and types of oils applied.

The preliminary findings reported here indicate that crude oil does affect the overall metabolic activity of microbial populations of the salt marsh. Further study is necessary to establish the mechanism of interference as well as the consequences of the reduction in select metabolic types.

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DISCUSSION

S. P. MEYERS: It occurs to me that maybe we're pushing our seed culture concept a little too hard at this time. A lot of concern about the Arctic deals with the sea ice itself, and the main problem in many of these polar seas would be the oil getting under the ice itself. There are reports, both from Antarctica as well as the Arctic, that the sub-ice contains a very active diatom population as well as other primary producers. These populations contribute considerably to the productivity of the areas. I wonder if we may be throwing out ideas about seed cultures, as far as the polar seas are concerned, that bear really no relationship to that environment. Some of the projections, for instance, are that the dissemination of any oil film, or spill, under the ice has about a 7-10 year transport time under the ice pack. Possibly Dr. Atlas would like to speak to this.

ATLAS: The work that I addressed myself to today was in terms of the Arctic summer. We are contemplating work when the bay is frozen over and I agree that the fate of under nearshore sea ice should be studied. The only person I know who has done any work in that area is Dr. D. K. Button of the University of Alaska, who, using radio-labeled hexadecane, placed some of the hexadecane under ice and trapped volatiles, presumably carbon dioxide. He found apparent degradation of the hexadecane under the sea ice during the winter months. Another person who has worked on the interactions of ice and crude oil is Dr. C. E. ZoBell, who found that when you had ice crystals present in a medium you actually increased the rate of degradation.

LEWIS BROWN: Showing the laboratory utilization of isolated hydrocarbons does not mean that they'll be used in crude oil. For example, we have used crude oil and related anthracene and added several fractions to it. With resting cells, if I remember correctly, we had to go to about 1000 ppm additional material before it was used in that total system in the presence of the crude oil. Still, the organisms will grow on a pure fraction. The question in my mind is whether many of these aromatics, or these potential carcinogens, etc., will be used under the environmental conditions that we're talking about in the open ocean.

R. J. MIGET: I think that environmental conditions in the open ocean are more conducive to oil degradation than the laboratory flask. In the oceans the more soluble low-boiling aromatic compounds will either evaporate or be greatly diluted in the water column--essentially fractionating into individual molecules. This situation is totally unlike laboratory studies where the more soluble hydrocarbons in crude oils (low-boiling aromatics) are forcibly concentrated in a relatively small volume of water within a closed flask. In this sense, then, I think that studies with particular pure hydrocarbons are quite valid.

There have been numerous studies (Boyland and Tripp, 1971, Nature 230:44-47; McAuliffe, Chem. Tech., Jan. 1971) which show that the alkylbenzenes I have used are the first ones to get into the water. In this same regard I would also like to refer to the work of Smith and MacIntyre (1971, Prevention and Control of Oil Spills, API/EPA/USCG, Washington, D.C.) on the initial aging of fuel oil films in the open ocean. They showed that a large percentage of compounds in crude oil boiling below 270 C were lost in the first six hours due to evaporation.

BROWN: I was looking at some of the low-level materials that potentially could be bio-accumulated. When you have a low level of the material you may not stimulate the population to such an extent that they would degrade. So, in a natural system I was questioning whether this would occur, although you can demonstrate these things rather adequately in the laboratory under artificial conditions. I'm not clear whether emulsification is a good or bad phenomenon, because there are disadvantages to both sides. If the oil is emulsified, our work has shown that it is much more toxic than if adsorbed to some material. At first thought, one might say that this is deleterious, if the concentration reached too high a level. On the other hand, if it's not emulsified, the oil has a tendency to pick up other materials that are toxic, like pesticides. Thus, as a concentrator of other toxicants, it might be better to emulsify the oil.

D. T. GIBSON: At the Atlanta meeting, the point was raised that we should be considering the higher molecular weight compounds in oil. I'm a little disappointed that so far we haven't had much discussion on these. It seems to me that if we are talking about anything that's going to be practical in the field, we have to come under federal regulations. It appears that chemical carcinogenesis by many of the compounds which are found in oil occurs after metabolic activation in the liver. The liver cells apparently fix one atom of molecular oxygen into compounds like benzpyrene or benzanthracene to make oxides which have been implicated as carcinogens. In a limited number of bacterial species that we've studied, both atoms of molecular oxygen are fixed and there is apparently no oxide intermediate. Fungi and yeasts that attack aromatic hydrocarbons appear to have a system similar to that found in mammalian liver. I don't see that there will be any approval to use fungi and yeasts unless we know a lot more about what some of these organisms do to some of these higher compounds.

And the other thing is the solubility of these aromatics. I don't think, as you said in the case of benzanthracene, that there are fewer organisms that are capable of degrading them. These compounds have zero solubility. If you could find a way to increase the solubility you could probably find organisms which would utilize them.

I have a comment in regard to Dr. Zajic's paper. I think I'm one of the men who reported growth of an organism on phenol above 100 mg per liter. In fact, we had no problem growing the bacterium up to 500 mg per liter.

R. J. MIGET: Dr. Ahearn, do you have any idea of the oxygen concentration in the sediments you were talking about?

AHEARN: All the yeasts we're working with are aerobes. They have the capacity to ferment, but they are not facultative anaerobes in the sense of bacteria. The yeasts will metabolize in an anaerobic environment but their actual growth is limited.

R. L. RAYMOND: I know of no valid experimental data that's ever been published in which oil was utilized without the presence of oxygen.

GERALD BOWER: A question for Dr. Rosenberg: What was the minimum amount of nitrogen and phosphorus needed for RAG-1 dispersion of crude oil?

ROSENBERG: Approximately 1 mM $(NH_4)_2SO_4$ and 0.01 mM K_2HPO_4.

BOWER: How much dissolved oxygen did you try to maintain in these tanks?

ROSENBERG: It wasn't measured. The air was run at 3000 liters/minute which was the maximum allowed us by the ship's Chief Engineer.

ALLEN LASKIN: I have a couple of comments rather than questions on a few of the points that Dr. Rosenberg made. I might take slight issue with his comment about the fact that microorganisms have had millions of years to develop oil emulsifiers. I'm a great believer in the power of microorganisms and their numbers and the length of time they've been around. However, bacteria may not have developed emulsifiers during these hundreds of millions of years in the same way we'd like to do it. There are, as you may know, some new chemical dispersants that are really quite remarkable in their activity in dispersing oil, the so-called second-generation, no-mix dispersants. So we may have a bigger problem competing with such dispersants if all we want to do is microbially emulsify oil.

I'd like to make one other point in relation to toxicity in developing sea urchins. This is a very neat system and it may give you quite a bit of information. It will tell you how the emulsified oil kills sea urchin embryos; however, it may not tell you how it kills fish or brine shrimp or algae. The toxicity of emulsified oil for gill breathers may well be related to particles of oil clogging the gills. So although we may know how oil affects nucleic acid synthesis or protein synthesis in developing sea urchin embryos, it may not give us all the answers we really need to have.

GIBSON: I think it's nice to see a field trial at long last. I wasn't clear how you inoculated your tank, Dr. Rosenberg. Did you use your isolated organism and, if so, what size of inoculum? Or did you use indigenous organisms that were already there?

ROSENBERG: We planned to inoculate it with 10^4 RAG-1 per ml. However, we ran into technical problems in transporting the inoculum to the ship. The indications were that we inoculated with only 10^2 RAG-1 per ml.

GIBSON: It seems to me from the data there's probably very little degradation of the oil. I wonder if we might run into the danger of "out of sight, out of mind" with this kind of thing at this stage of development.

ROSENBERG: That's an excellent point. In this initial ship experiment, we did not degrade more than 10% of the crude oil. Our long-range goal, however, is more extensive breakdown and recovery of the products on board. However, if you have a present choice of putting dispersed oil or crude oil that's not dispersed into the open sea I would recommend dispersed oil. It has less chance to get to where most of the marine life is concentrated: it has much less chance of aggregating and forming tar on the shore, and most importantly, because of the large dilution there should be much faster microbial degradation in the sea.

P. A. LaROCK: First, a comment on your technique. I think this type of process has certain industrial applications obviously for cleaning vessels, but perhaps instead of just releasing this emulsified material from the holds, it could be passed through an oil-water separator or held until the vessel got to port. In this way, additional oil sludge would not be released. It could be returned to the refinery and processed with other oils or other cargoes.

Secondly, a comment on dispersants in general. We find that certain bacterial species are affected by certain aromatics in the ppb range. What would be the effect of dispersants or emulsifying agents on the persistence of these aromatics, some of which may normally evaporate? If you emulsify them and disperse them, you may do more harm to phytoplankton production or microbial activity.

RAYMOND: I would like to correct something Dr. Rosenberg has said about the emulsification capabilities of his microbes exceeding the chemical systems. I believe that if Vern Coty were here, he'd take a great exception with you. I think we need also to correct the impression that we have to develop new great strains of microorganisms. Those of us in the field for many, many years know that these are around. What we have to do is to alter the environment so that the organisms can grow on the substrate that's available to them. You are not going to find a universal panacea in any one microorganism for any

one situation in this world. Just because we have a nutritionally balanced system in the Delaware River, say, or in the Marcus Hook Refinery, that system doesn't prevail in Tulsa, Oklahoma, or Los Angeles, California. Each situation has to be studied on its own merits. It's fairly simple, I think, to compute the amount of nitrogen needed to use almost any hydrocarbon. I take the universal figure as a "I'm going to have, for every 100 lbs of hydrocarbon, about 6 lbs of nitrogen." It's got to come from somewhere. You have to have at least a ton of oxygen for each ton of hydrocarbon to convert to cell material. There's no way this can be avoided. This must be considered. If you have a body of water and you have 100 or 1000 tons of oil, it would take a lot of nitrogen to convert all that oil into cell material.

ROSENBERG: From the practical point of view, in a closed system, nitrogen is not the problem. In other words, if you compute the cost and savings, and you talk to people here who know about this, the major cost is the air, and how to get the air. Cheap enough fertilizers are available. In an open system which may require special kinds of nitrogen, then cost might be excessive.

AHEARN: One of the interests in developing seed culture systems is possibly to increase the rate of degradation of the entire crude oil complex, by increasing the surface area. So strains that have the capability of emulsifying the substrate as well as utilizing a significant fraction of it would be of extreme value. In fact, one of the questions that did come up earlier is, how common are strains that will show good utilization and good emulsification? One of the problems that we've had in our work is finding these two characters together in the same strain. I would like to ask Dr. Crow, if he would, to give us a comment on the percentage of organisms with both these properties that he has found in his surveys.

S. A. CROW: We've studied approximately 50 isolates that all utilized hydrocarbons fairly well. Emulsification was a fairly rare property in the organisms we were working with. We've done all our work in the estuarine environment and less than 5% of our isolates showed any emulsification of crude oil. I think this is fairly significant--it shows it's not a real common property.

ZAJIC: I only have one comment. In our experience most of the organisms isolated using oil enrichments have an emulsification property. Thus it must have something to do with how you isolate the cultures. In my experience, emulsification is a very common phenomenon in microbes degrading oil. In the early work completed on kerosene, we used a kerosene enrichment. Eighty-four cultures were isolated in pure form that would grow on kerosene and each was tested for its emulsification capability on kerosene. Whereas many of them emulsified kerosene, only four produced quantities of an emulsifying agent that would be regarded as

interesting. Of these, only four had industrial potential.

AHEARN: I've been trying to find out from different workers the incidence of emulsifying strains. It seems that when you study chronically polluted sites, or if you look at strains from oil fields, you more commonly find organisms which emulsify as well as utilize various fractions of the oil. Dr. Crow's work has been in an area which has been remote from direct oil pollution, or chronic oil pollution, and his results agree with what we have found. When we have looked at oil polluted sites we have found a fairly high percentage of the isolates of one or two species which emulsify oil. But these represent only a minority of the species isolated. Our culture systems have used both enrichment and general isolation procedures.

ZAJIC: I would make another comment, that is, we have been using some of the aromatics for enrichment purposes. In so doing, a much lower percentage of microorganisms produce emulsifiers. So I think most of the emulsifiers observed in complex systems are from the paraffinic hydrocarbons, and the emulsification property will be greatly enhanced by the presence of paraffins.

RAYMOND: Dr. Zajic and Dr. Rosenberg both, I believe, mentioned that you don't find these organisms prior to seeding. The technique that is used in looking for these organisms must be considered. If you use conventional enrichment techniques, for instance, you will isolate the organisms. Numerous production reports mention the problem of emulsification of oil. Every producing oil field has probably had to worry about breaking the emulsions that bacteria cause in these systems. If you study a soil which is virgin to oil, you can't use the same isolation techniques that are used when you work in a system that has oil contamination. But the organisms are going to be there. You have to look at a tenth of a gram of soil instead of 1-10,000 or 1-1,000,000 dilutions and different techniques are necessary. You don't have to develop microbial seed systems--they're there.

ROSENBERG: Both of us were not arguing that these bacteria were not there. The gist of the Zajic talk was that if you choose conditions right, you can by definition of enrichment culture enrich for the bacteria you particularly want. If you want to add it back later in a closed system or an open system, you can do so. But I don't think either of us intended, in any sense, to infer that these bacteria aren't there. The whole definition of the enrichment culture is to select conditions to enrich for a particular kind of desired microbe. Certain enrichment cultures turn up more emulsifiers while others turn up other things. I selected one organism on purpose by transferring the enrichment culture some 8 or 10 times and subjecting it to conditions where I selected the fastest grower under a particular set of conditions. I eliminated, purposely, many other bacteria.

ARTHUR KAPLAN: In our experience with refined products, JP-4, in a military fuel distribution system for aircraft, we found that emulsification with the bacteria was quite common. I can't recall an instance where the fungi gave us any emulsification and I don't recall the situation with the yeasts.

CONCLUDING REMARKS

Dr. William Upholt
U.S. Environmental Protection Agency
Washington, D.C.

The EPA has the responsibility of regulating all materials sold or used to manage pest populations as well as to protect man and his environment from all types of pollutants. This is the basis for EPA sponsorship of this important conference. We are especially appreciative of the insights that the participants in the conference have given us into these problems of the use of microorganisms as you have discussed them for the past two days. We are impressed, as I'm sure you are as well, by the difficulty of protecting against a hazard that is actually unknown. In the past we have registered some chemical pesticides, only to discover later on that they created hazards that we had not anticipated. In some of those cases the unanticipated hazards have led us to cancellations and very expensive and sometimes overly emotional public hearings. We would hope to prevent these types of mistakes as microbial pesticides are submitted for registration and tolerances or exemptions from tolerances. Obviously, it is to everyone's advantage if we can provide guidelines on the needed information for registration as early as possible. We are in the process now of issuing guidelines for registration of chemical pesticides, but they are hardly adequate for microbials. As most of you know, we have suggested preliminary guidelines for nuclear polyhedrosis viruses but even those are of limited value with other types of viruses and with bacteria, fungi and protozoans. Frankly, it is difficult to write guidelines for submittal of information regarding an unknown hazard. Of course, we, like all of you, hope that there is no hazard to man or to other non-target organisms from these microbial pesticides. Nevertheless, there are obvious potential hazards, such as infectivity to man, domestic animals or beneficial non-target organisms. These hazards are doubtless greatest to those non-target organisms which are most closely related to the target organism. Information to this effect would be essential for registration. Beyond that, discussions during the past two days have suggested possible allergic reactions in man, or even some type of highly atypical infection, perhaps by a fungus. Such atypical reactions are very difficult to predict, and therefore difficult to evaluate. As also was pointed out in several comments in the last day or so, negative results are extremely difficult to evaluate. They are never any better than the adequacy of the tests that produce them. This is doubtless the reason, or at least one reason, that editors hate to accept for publication these

negative results. It's also the reason that we hesitate to accept them as a basis for registration. We register on the basis of balancing benefits against risks. Unless you know the hazards versus the risks, it's difficult to get an objective evaluation of such unknown risks. In this situation, we will doubtless make full use of the stepwise procedures which permit registration for experimental purposes prior to full-scale commercialization. During this period and later on, monitoring will be necessary and extremely crucial to the procedure. We will need the full cooperation of all of the experts throughout the country to design adequate guidelines for registration and monitoring during and after the experimental stage, if we are going to successfully protect our society from the unanticipated hazards from these microbial pesticides. We are anxious to have these new materials available for use but as you know society takes a dim view today of providing 200 million guinea pigs. Society is demanding more cautious approaches to all hazards. We are sure that if we all work together, we can provide that needed assurance of safety as well as efficacy within a reasonable time. We therefore do appreciate the opportunity to participate in this conference and we will be calling on you more and more in the future to help us set up the guidelines and decide what is needed for our full registration.

III

SUMMARY PAPERS

BACTERIA: SUMMARY

Anopheline and Culicine mosquitoes are the target invertebrates in aquatic environments for which species of *Bacillus* are considered to be used as control agents. The principal entomopathogenic bacterial species are: *B. thuringiensis*, *B. sphaericus*, *B. moritai*, *B. cereus* var. *juroi*, and varieties within the *B. alvei circulans* morphological Group II. Of these, only *B. sphaericus* is currently considered specific for mosquitoes. Each *Bacillus* species should be considered as a source for selection of strains with specific activity for target aquatic invertebrate pests.

Preliminary laboratory evaluations of *B. thuringiensis* (BA068) and *B. sphaericus* have been completed in the United States, the latter also in Nigeria. Additional laboratory tests and pilot small plot field trials are anticipated for 1974 at locations within and outside the United States.

Commercially available preparations of *B. thuringiensis* are limited in their activity to Lepidopteran insects. Mice in standardized laboratory safety tests and fish from aquatic environments have not been affected by *B. thuringiensis*. *B. sphaericus* appears to be limited in activity to culicine and anopheline mosquitoes. Additional screening has not demonstrated activity in the house fly (*Musca domestica*) or a single species in each of the orders Lepidoptera and Coleoptera.

Documentation is not available to describe either the persistence of the entomopathic bacilli in aquatic environments or the results of direct or indirect introduction of bacilli to aquatic systems.

A multi-stage review system for biological agents anticipated by the vector biology and control unit (VBC) of the World Health Organization (WHO) should provide specific information of impact of bacteria in environments and associated water systems.

RECOMMENDATIONS

1. Investigate:
   a. persistence of bacilli and their products in aquatic environments.
   b. safety of *B. sphaericus* for non-target organisms as a model for similar bacilli.
2. Support programs for detection and development of useful microorganisms for management of target invertebrates in aquatic environments.

Summary prepared by: S. Singer (presiding), J. Briggs, S. R. Dutky, A. M. Heimpel, E. W. Davidson, T. L. Couch, T. C. Cheng.

VIRUSES: SUMMARY

Virus diseases are known to affect many invertebrate species living in aquatic habitats. These include the nuclear polyhedrosis types (NPV), e.g. *Baculovirus*; cytoplasmic polyhedrosis viruses (CPV); entomopoxvirus (EPV); and non-inclusion viruses (NIV), e.g. *Iridoviruses*. None of these has been studied sufficiently to recommend a specific virus as a control agent for the aquatic insect pests.

Laboratory and field studies have concentrated on viruses of mosquitoes. The *Iridovirus* from *Aedes taeniorhynchus* and the *Baculovirus* from *Aedes sollicitans* have received the most attention. Both of these viruses have been studied in the laboratory in attempts to quantity and determine levels of infectivity and susceptibility. Limited host specificity studies also have been made. Cross-infection tests with the mosquito iridescent virus (MIV) from *A. taeniorhynchus* have been carried out against approximately 18 species of mosquitoes, 3 species of Lepidoptera, and 2-3 additional species of Diptera. Present testing indicates that MIV appears to be restricted to the floodwater genera of *Aedes* and *Psorophora*. Safety testing against aquatic species has not been done with any viruses from aquatic insects. Limited studies have been made with the NPV from *A. sollicitans* against mosquito predators such as *Gambusia* and insects of the families Hydrophilidae, Dyticidae, and the order Odonata. Preliminary cross infectivity tests indicate that this NPV is restricted to mosquitoes of the genera *Aedes* and *Psorophora*.

Studies on the persistence of virus in the aquatic environment have not been made. Similarly, there is no available information regarding possible effect of metabolic by-products from viral infections on aquatic organisms.

Field related studies with MIV from *A. taeniorhynchus* and NPV from *A. sollicitans* (1 test with each virus) have been reported. It is obvious from the previous assessment that commercially available virus preparations for use in control of aquatic insect pests are not available.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. There is an urgent need for intensive surveys to discover additional potentially useful viruses. Research in this area should be closely coordinated at national and international levels.
2. It is highly important that research be greatly expanded on the presently known viruses. This research should include biochemical-biophysical characterization and identification, studies on infectivity, host specificity, and safety; and production of viruses for possible field use.

Summary prepared by: D. W. Anthony, T. B. Clark, A. M. Heimpel, J. D. Paschke, M. D. Summers, J. N. Couch.

FUNGI: SUMMARY

At present there are no fungal pathogens of invertebrates employed in wide-scale pest control operations which directly involve aquatic habitats. This could change within the next few years. Laboratory and exploratory outdoor tests for larval mosquito control are now underway with *Coelomomyces* spp., *Lagenidium giganteum*, *Beauveria tenella*, and *Metarrhizium anisopliae*. Field observations indicate that some species of *Entomophthora* offer promise as pathogens of adult *Culex*.

The current lack of detailed information on proper timing, dosage, and formulation, in addition to generally inadequate host-range data, poses serious difficulties in estimating the impact of most entomogenous fungi on aquatic environments.

None of the above species are commercially available but the technology exists for mass production of all except *Coelomomyces*.

Laboratory infections have been readily obtained with all except *Coelomomyces*. Small-scale field studies have been conducted with *Coelomomyces*, *Lagenidium*, *Beauveria*, and *Metarrhizium* with promising results.

Meaningful safety tests of *Coelomomyces* are not possible at present, because the infective unit of the fungus is unknown. Field collections indicate these fungi have narrow host ranges, usually one or a few mosquito species. One isolate of *L. giganteum* has a wide mosquito host range but apparently poses no threat to other organisms in mosquito habitats. Specific laboratory tests with a wide variety of aquatic and terrestrial organisms were negative. Other isolates of *Lagenidium* are little studied, but at least one is reported as infecting *Daphnia*. The majority of the *Entomophthora* currently under investigation appear specific to *Culex* adults. A wide range of target species are susceptible to the *B. tenella* isolate from mosquitoes; however, its infectivity for non-target species is unknown. The isolate of *M. anisopliae* under consideration is more virulent for mosquito larvae than most other aquatic invertebrates tested, but certain aquatic organisms were susceptible. All major mosquito groups were susceptible. Toxins are unknown from *Coelomomyces* and *Lagenidium* and tests on those metabolites produced by the other fungi under consideration, although incomplete, have not indicated any serious problems.

Coelomomyces spp., *L. giganteum*, and possibly *B. tenella* will probably become established when properly introduced. *M. anisopliae* does not produce new spores on submerged cadavers, so it is not likely to become established as a self-perpetuating mosquito pathogen after artificial introduction.

Conidia of *Beauveria bassiana* are commercially available in the USSR for use against orchard and field pests. Field trials are being conducted in France with *B. tenella* against subterranean beetle larvae, and in the United States *Hirsutella thompsonii* and *Entomophthora*

thaxteriana are being tested against orchard mites and row crop aphids, respectively. The amounts of these pathogens which may find their way into aquatic environments following application to terrestrial environments is unknown. *B. bassiana* has a very wide insect-host range, but testing with aquatic organisms is meager. Allergic reactions to this fungus have been reported. *H. thompsonii* and *E. thaxteriana*, in limited safety tests, were safe for vertebrates. Field observations indicate both have relatively narrow insect-host ranges, but neither has been tested against aquatic invertebrates.

Summary prepared by: D. W. Roberts, E. M. McCray.

PROTOZOA: SUMMARY

Only one microsporidan, *Nosema algerae* Vavra and Undeen, is presently being considered for field tests against aquatic insect pests. This microsporidan has been shown to cause high mortalities in many species of *Anopheles* mosquitoes in laboratory infection studies. Its ability to produce mortalities in field populations of *Anopheles albimanus* was demonstrated early this year by insect pathologists from the Insects Affecting Man Research Laboratory, USDA-ARS, at Gainesville, Florida, in cooperation with the Army in the Canal Zone. Several other undescribed species recently found also have been shown to cause mortalities in mosquitoes in laboratory tests, and some of these may be considered for field testing in the future. No microsporidans are presently being seriously considered in tests against ceratopogonids, simulids, tabanids, or other aquatic pests.

Viable *N. algerae* spores were fed to numerous non-target animals by researchers at the Gainesville Laboratory to determine host specificity of this microsporidan. Some animals tested were: amphipods, aquatic predaceous hemipterans, cabbage loopers, chickens, chironomids, cockroaches, corn earworms, crayfish, damsel flies, dragon flies, dytiscids, fire ants, fish, fresh-water shrimp, helgramite, house flies, and mice. Only three (chironomids, corn earworms, and house flies) were susceptible. Under natural conditions, however, only the chironomids are likely to be exposed to *Nosema*.

Spores of *N. algerae* are unstable in water at room temperature and even lose viability when stored for more than six months under refrigeration. Drying immediately kills spores, making it even more unlikely that they will infect terrestrial animals. Accumulation of *N. algerae* spores in aquatic environments should not be a serious problem. Spores not ingested by mosquitoes rapidly lose viability. Microsporidans do not produce toxic metabolites.

Development of *N. algerae* was obtained in mammalian tissue cultures of kidney cells at 26 and 35 C, but development could not be completed at 37 C. Small numbers of meronts, sporonts, and sporoblasts were observed at the sites where large numbers of *N. algerae* spores were subdermally injected into low temperature regions of mice, i.e., base of the ears, tail, and hind feet. Spore-to-spore development was not confirmed, and the protozoan was confined to the injection site. None of the stages of *Nosema* were found at the injection sites 12 days after injection.

No mice or other vertebrates were ever affected by massive dosages of spores per os by by intravenous or intraperitoneal injection of spores. Development was never complete at temperatures at or above those of warm-blooded animals in tissue cultures.

The studies indicate that the host range of *N. algerae* can be extended experimentally to include a large range of hosts; however, under

normal field conditions, these routes of infection would not normally be available.

There may be a remote possibility that microsporida may enter non-host warm-blooded animals through injury sites or punctures produced by mosquitoes or biting insects. Present information indicates that these introduced microsporida would be contained at the site of entry and would be destroyed. However, such possibilities should be investigated in conjunction with safety tests on pathogenic microsporida in mosquito and biting flies. All other microsporidans that may be considered in future field tests should be evaluated for safety to man and the environment utilizing guidelines produced by the Environmental Protection Agency before they are used in large-scale field tests.

Summary prepared by: E. I. Hazard, J. E. Henry, J. Maddox, A. Undeen.

PERSISTENCE IN AQUATIC HABITATS: SUMMARY

Knowledge of persistence of insect pathogens in the terrestrial environment has increased substantially. However, that concerning the aquatic environment (both fresh water and marine) is still very limited. This situation demands immediate attention, because a number of pathogens are now showing real promise for control of aquatic insect pests and vectors.

Investigations of persistence should consider both biotic and abiotic factors of the environment. Pathogens of not only aquatic insects but also terrestrial ones with potential for control of aquatic pests should be studied in this context. Major abiotic factors that should be considered are salinity, temperature, water depth and movement, desiccation, redox potential, soluble materials, pH, turbidity, and the sediment of the aquatic environment. In the biotic environment, the primary and secondary hosts, animal carriers, and other microorganisms should be considered. This will involve studies on interspecific transmission as well as on persistence in the digestive tracts and on body surfaces of animal carriers. In addition, the interactions of hosts and pathogens with other aquatic fauna and flora should be investigated.

Relevant laboratory studies are important, but the ultimate assessment demands aquatic-field studies. Persistence of pathogens that are highly mobile, because of buoyancy or motility, require investigation in both fresh-water and marine environments. The studies to be conducted will be determined primarily by the type and nature of the pathogen.

Other speakers have enumerated pathogens of mosquitoes and black flies which offer the best potential for development. Due to the advanced state of investigations, the following are suggested as candidates for immediate studies of persistence in aquatic environments: nuclear polyhedrosis viruses; *Bacillus sphaericus*, *B. thuringiensis*, (var. BA068); *Coelomomyces* spp., *Lagenidium giganteum*, *Beauveria tenella*, *Metarrhizium anisopliae*; *Nosema algerae*; *Reesimermis nielseni*. Studies on persistence should consider: control of the target pest, safety to man and other terrestrial vertebrates, pathogenicity for non-target aquatic invertebrates and vertebrates, and pollution from the standpoints of health and aesthetics.

Summary prepared by: H. C. Chapman, R. B. Jaques, M. Laird, Y. Tanada.

MICROORGANISMS TO CONTROL AQUATIC PESTS OTHER THAN INSECTS: SUMMARY

To our knowledge, no extensive research program is currently underway in this country or abroad to isolate and develop microorganisms for biological control of aquatic pests other than insects, although there are occasional reports of organisms that may be potentially pathogenic to aquatic snails that serve as intermediate hosts for the human-infecting schistosomes. Such organisms, however, are currently not being maintained in culture in any laboratory. *Bacillus pinottii*, a Gram-variable species, was thought at one time to be useful for the control of *Biomphalaria glabrata* in Egypt and Venezuela; however, its pathogenicity to this gastropod vector of *Schistosoma mansoni* has been seriously questioned.

At Lehigh University's Institute for Pathobiology, a yet unidentified coccus has been isolated from Israel from the desert snail *Theba pisana* which is lethal to this gastropod when the ambient temperature is lowered to 10-18 C. The usefulness of this bacterium against other species of molluscs, however, remains to be ascertained.

Despite the lack of rigor in efforts to search for biological control agents for aquatic pests other than insects, the potential usefulness of microorganisms for this purpose should not be underrated. Support for goal-oriented basic studies that will permit development of a rational approach to isolation, selection, and development of control agents against toxic marine invertebrates and disease-carrying freshwater animals, especially molluscs, must be forthcoming from agencies with vision.

Summary prepared by: T. C. Cheng.

PLANT PATHOGENS FOR CONTROL OF AQUATIC WEEDS: SUMMARY

Studies on the evaluation of plant pathogenic biocontrols of water weeds are in their infancy. However, recent explorations on the use of plant pathogens to control aquatic weeds have been encouraging. Two related aquatic systems subject to weed problems have been studied. In one, consisting of a total aquatic ecosystem, four exotic aquatic weeds--water hyacinth, hydrilla, alligator weed, and Eurasian water milfoil--have been the target of our studies. The second is a semi-aquatic agroecosystem in which a weed (*Aeschynomene virginica*) that occurs in rice has been the subject of extensive and successful field tests.

Several foliar fungal pathogens of water hyacinth have been tested for pathogenicity, host range, and efficacy as biocontrols under laboratory conditions. Of these, *Cephalosporium zonatum*, which causes zonal leaf spot of water hyacinth, is the most likely candidate for control of this weed in Florida. When used, its effect on water hyacinth is likely to be increased due to the presence of two arthropods (*Neochetina eichhorniae*, weevil; *Orthogalumna terebrantis*, mite) already in use as biocontrol agents of weeds in Florida. *Cephalosporium zonatum* is present in Florida and Louisiana on water hyacinths. Therefore, special plant quarantine regulations will not be needed for release of this organism for field tests. Laboratory tests on the host range of this pathogen are in progress. Numerous crop and non-crop plants are being screened. Limited field tests are underway to determine efficacy of this pathogen under natural conditions and its effects on non-target hosts. Studies have not been done on possible toxicity or pathogenicity of *Cephalosporium zonatum* to fish, other aquatic animals, terrestrial invertebrates and vertebrates; persistence and survival in the environment; and impact of its use on the "total ecosystem."

Available evidence suggests that in the absence of suitable hosts like water hyacinth, *C. zonatum* will lose viability in aquatic habitats. Long-term persistence of this organism in water is therefore not anticipated.

Other pathogens of water hyacinth that are currently being evaluated include *Alternaria eichhorniae*, *Cercospora piaropi*, a *Helminthosporium* sp., (*Drechslera* sp.), and *Rhizoctonia solani*. Several isolates of *Penicillium*, *Aspergillus*, and *Trichoderma* that are toxic to hydrilla are also being studied to identify possible host-specific phytotoxins. Attempts are being made to seek newer pathogens of water hyacinth, hydrilla, alligator weed, and Eurasian water milfoil.

The success in controlling *Aeschynomene virginica* (northern jointvetch), a semi-aquatic weed of the rice fields, with the fungal pathogen *Colletotrichum gloeosporioides* signals the entrance of plant pathogenic bioherbicides in the weed control scene. Laboratory and field tests indicate that this pathogen is specific to *Aeschynomene*. Out of

150 plant species screened, only *Aeschynomene* was susceptible.

Preliminary toxicological studies indicate that this fungus is non-toxic to warm-blooded animals. Research on this extremely host-specific, endemic fungus has advanced to a stage where its commercial production and large-scale aerial application are being seriously considered.

Summary prepared by: R. Charudattan, G. Allen, G. E. Templeton.

SUMMARY COMMENTS

C. M. Ignoffo

It is rather obvious that the status of aquatic microbial pesticides is still in the formative stage. I think it far-sighted of EPA to take the initiative in arranging a conference such as this to document the baseline now in an attempt to aid future developments and, again, historically to measure the developments that will undoubtedly occur in the future. Now the meeting scope was limited to aquatic habitats. This major theme meant by its very limitation that the successes and developments of microbial insecticides for terrestrial pests could not be covered. I want to put this in its proper perspective now, to give you some idea of the extent of developments that have occurred in this terrestrial area.

There are presently about 40 trade-name microbial insecticides in the world. These are based on approximately 12 pathogens, representing groups of bacteria, fungi, and viruses. These materials have been used on every major continent except one--and I'll let you guess which one that might be. One commercial bacterial insecticide, which is used to control the Japanese beetle, has been introduced in about dozen states, in the northeast, and since 1950 has exerted control over an area estimated at a quarter of a million square miles. Another bacterial insecticide is used on cabbage, lettuce and many cold crops in the United States, and has been used since 1959 at an estimated rate of about 2-3 million acre treatments per year. In Russia, approximately 10 percent of their total insecticidal control, perhaps 45 million hectares, is devoted to biological control, with at least 50% of this being microbial insecticide applications. In Japan, a commercial viral insecticide has been developed that is used to control a pest of pine forests. Now, hopefully, these figures and facts will help you to better understand that microbial insecticides have been developed, from concept, from isolation in the field, to commercialization. Associated with this development has been a vast accumulation of basic information on pathogen, host and ecosystem. Microbial insecticides are not just laboratory curiosities. They are safe, they are selective, they are efficacious, they are commercial products currently in use as pesticides.

MICROBIAL DEGRADATION OF OIL: SUMMARY

The use of microbial seed systems to facilitate the biodegradation of oil or other hazardous chemicals is in a primitive state of development. Indeed, there is some controversy that fertilization to stimulate the indigenous microorganisms might not provide the most efficient biodegradation. It is questioned also if either procedure is applicable to open systems. Still, preliminary studies, as presented and discussed here, indicate that fertilization or seed systems, or a combination of both, are presently being used or explored. It is possible that both types of procedures may eventually be found useful for varying goals or ecological conditions.

Generally, those here agree that there are insufficient data to fully evaluate the effectiveness of these seed or enrichment systems in degrading a target substrate. Moreover, there are few data available to permit an assessment of potential hazards of seed systems to the environment. Guidelines for the control of seed or enrichment systems should consider the relative or potential hazards of the process and target systems. The present status of the art requires us to proffer only general guidelines. As a committee we suggest that no established pathogens for man be added to the environment to treat oils and/or related products. It should be emphasized that treatment procedures being proposed are directed toward acceleration of natural processes.

Summary prepared by all participants of Session II.

REGULATORY ASPECTS OF MICROBIAL PESTICIDES

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Although there are broad and common phrases used by the Environmental Protection Agency to state its mission and its goals--such as to "protect man and his environment" and to assure that pesticides are used in a "safe and effective manner"--any regulatory agency also has the responsibility to encourage the research or effort leading to these goals, to fulfill its mission. Thus, as evidenced by this symposium-workshop, the Agency is looking to alternate methods of pest control to replace hazardous, toxic or persistent chemical pesticides. The Agency encourages, through support of research under contract, through its own laboratories and various cooperative efforts with industry, academia, and other Federal agencies, the development of alternate methods of pest control and the implementation of the concepts of biologically integrated pest management. Section 20 of the 1972 Federal Environmental Pesticide Control Act (FEPCA) points out that priority must be given to this type of pest management research.

But we also must remind everyone of the regulatory aspects of the Agency and the intent of Congress in establishing the Environmental Protection Agency in the first place. We do have regulatory responsibilities under FEPCA, Food, Drug and Cosmetic Act, the Water Acts, etc.--particularly when a product reaches the open market. These responsibilities relate to the safe and effective use of products intended to destroy, control, or mitigate various pests--some of which have been the primary focus of attention at this symposium. In the past some chemical pesticides were registered only to discover later that they created hazards that had not been anticipated. In some cases these unanticipated hazards have led to cancellations and very expensive and sometimes overly emotional public hearings. Such mistakes should be prevented as microbial pesticides are submitted for registration and tolerances or exemptions from the requirement of a tolerance.

We must ask the question, "What could possibly happen?" when using microbial pesticides. But we also should qualify the question by asking, "How likely is it to happen?" The scientific search for answers to these questions should eventually fill in the voids that are apparent in our knowledge about these new generation pesticides.

It is to everyone's advantage if EPA can provide guidelines on needed information as early as possible. We are in the process now of issuing guidelines for registration of chemical pesticides but they are hardly adequate for microbials. Preliminary guidelines for nuclear polyhedrosis and granulosis viruses are nearing completion but even these are of limited value for other types of viruses, bacteria, fungi, and protozoans. It is difficult to write guidelines for submittal of information regarding an unknown hazard. Because of the multitude of issues involved, experts in the fields of microbiology, epidemiology and public health must cooperate in order to arrive at the best possible guidelines for registration and tolerance setting as well as for monitoring programs during and after the experimental use. We hope that there is no hazard to man or other non-target organisms from these microbial pesticides, but that does not seem to be enough to make a final decision. There are obvious potential hazards such as infectivity to man, domestic animals, or beneficial non-target organisms. These hazards are doubtless greatest to those non-target organisms most closely related to the target species. Beyond that there is a possibility of allergic reactions in man from some exo- or endotoxin that has not been detected, or some type of highly atypical, unpredictable infection from a fungus perhaps in lungs or possibly other organs.

The eventual full-scale use of microbial pesticides must follow a carefully planned, stepwise approach and the issuance of experimental use permits under Section 5 of FEPCA will be an important aspect of their development, which can be briefly summarized as follows: (i) Identification of the pathogen, including growth requirements, stability, bioassay and infectious process. (ii) Preliminary determination of usefulness. (iii) Effects on non-target organisms, acute, subacute and long-term studies. (iv) Review of aspects of safety. (v) Small-scale use to gather data on efficacy as well as to monitor effects on the environment. (vi) Second, comprehensive review of safety aspects and performance under field conditions. (vii) Extended use, with continuous monitoring of environmental effects. The monitoring programs will be of special importance. Good ecological baseline data are needed in order to assess effects of the deliberate use of pathogens, and humans exposed to the pathogens during production and use must be monitored for any adverse reactions.

Many, if not all, of the safety tests will give negative results which are extremely difficult to evaluate since it is axiomatic that it is impossible to prove a negative. The adequacy of the tests therefore must be scrutinized as much as the actual results. The empirical tests, although they probably will never be completely replaced, should be complemented at an increasing rate by basic knowledge about the mechanisms which make an insect (or plant) pathogen as selective as we hope it is.

It is important that we have addressed these questions today and we are anxious to have the candidates available for use. Society demands a cautious approach to all potential hazards and takes a dim view of providing 200 million human guinea pigs. Large commercial endeavors

are expected to occur only in 3-5 years and this time should be used to gain progressively more knowledge concerning the safety as well as efficacy of microbial pathogens. More interest and collaboration in this area of pest management must be stimulated, and this can best be done by supporting applied and basic research, by conducting symposia and workshops but more importantly by cooperative efforts on the national (USDA-EPA-Industry) as well as international (WHO/FAO) level.

CONCLUDING REMARKS

Dr. John Buckley
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Really, the only reason I accepted this was that it gives me a chance to say something after Bill Upholt. In all seriousness, I suppose I want to add my thanks but I want to direct them the other way. I missed most of this meeting because I attended another one. I heard enough of the discussions both at the laboratory last evening and then this morning to know that it must have been an extremely stimulating time. I'm sorry indeed that I couldn't have been here for it. I'd like to thank each of you, then, for coming and talking and discussing because it seems to me that several things seem clear to me. One of them is that all of us in the room, I believe, have an inherent feeling that the use of organisms that are already present in the environment to do something that we want done in the environment is likely to be safer than the introduction of exotic chemicals to perhaps do these same things. Dr. Upholt touched on this a bit when talked about the weighing of benefits and risks. And the last discussions that just came up highlighted, again, the problem of non-pathogenic or safe--we really shouldn't write those words without quotes around them. Pathogenic to what and with what frequency and under what circumstances? Or safe, to what again, to one in a hundred million, to all but one in a hundred million, to half the population? There are enormous questions bound up in this and we try to summarize our thoughts on it, we tend to treat things as absolutes, as being one way or the other. In reality, the kinds of things we need to deal with are almost all in some kind of miserable shade of gray that won't fit neatly into "safe" or "unsafe," "pathogenic" or "non-pathogenic," or any of these other words we like to use. I was fascinated this morning by what seemed to me a very concise state-of-the-art review, particularly in the insect pathogen area. I know that something is going on in the oil area. But it seemed to me that discussions of the sort that you have been having have been very worthwhile and let me add my thanks to all that other pile of thanks.

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IMPACT OF THE USE OF MICROORGANISMS ON THE AQUATIC ENVIRONMENT
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Proceedings of Workshop-Symposium held at Pensacola Beach, FL, April, 1974

16. Abstracts This report contains the proceedings of a symposium-workshop sponsored by the EPA Gulf Breeze Environmental Research Laboratory to determine the possible impact of artificially introducing microbial insect control agents or oil-degrading agents into the aquatic environment. The efficacy and safety testing, especially against non-target aquatic organisms, for use of bacteria, viruses, fungi, and protozoa to control aquatic insect pests is discussed with remarks of panel members representing government, academia, and industry. Special attention is given to persistence of pathogens in aquatic environments as well as control of aquatic weeds and other non-insect pests.

The use of microorganisms to clean up oil spills in aquatic environments is discussed by industrial, academic, and governmental scientists. Special considerations are given to selection of hydrocarbonoclastic microorganisms and use of these microorganisms in special environments--Arctic regions and Louisiana salt marshes.

17. Key Words and Document Analysis. 17a. Descriptors
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17. Key Words and Document Analysis.

aquatic systems	viruses	pathogen persistence
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