ENCLOSED SYSTEMS FOR TESTING MICROBIAL PEST CONTROL AGENTS

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Office of Pesticide Programs and
Office of Research and Development

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ORGANIZATION OF WORKSHOP

(WORKING GROUPS AND EFFORTS)

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Chairman - Ray Seidler	Chairman - Dick Anderson	Co-Chairman - Tom Duke	Chairman - Clint Kawanishi
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INTRODUCTION

by

John A. Couch

Background

This report stems from a workshop held at the EPA, Environmental Research Laboratory, Gulf Breeze, Florida on February 18 and 19, 1986. The workshop and report were requested by the Hazard Evaluation Division of the Office of Pesticide Programs.

The report consists of descriptions and documentation of some enclosed, multispecies systems that may be used for laboratory testing of both natural and genetically altered microbial pest control agents (MPCA's-viruses, bacteria, fungi, and protozoa) for possible effects in nontarget species, and ecosystems.

Laboratory tests systems relative to estuarine, freshwater, and terrestrial habitats, related species, and physical-chemical factors were considered by the members of the four working groups (p. ii) and the workshop as a whole. Each working group wrote a section of this report relevant to the needs for risks evaluations appropriate to their respective ecosystem types (Sections III, 1, 2, and 3).

Goals and Attributes of Test Systems

The purpose of this report is to provide specific recommendations which would permit properly trained biologists to construct an enclosed, laboratory system to evaluate the effects of microbial pest control agents (MPCA's) at several levels of biological complexity. These described systems may provide the opportunity to examine possible effects of MPCA's in nontarget species in regard to ecosystem function as it may be altered by such phenomena as gene transfer, persistence of agent, microbial metabolic perturbation, or

changes in population and species compositions due to unexpected competition or replacement. Other, very important endpoints of great concern, centered below the ecosystem, species or population levels may also be studied in the suggested systems. These primary effect endpoints, such as infectivity, pathogenicity, and toxicity are expressed via the modes of action of most MPCA's and may be studied in individuals of nontarget species selected to inhabit the enclosed systems. Therefore it may be feasible to study possible activity of MPCA's at several biological/ecological levels in these suggested test systems.

The attributes of the test systems desired include:

1. Containability[1]
2. Reproducibility
3. A variety of trophic levels available in one or more of the systems
4. Reasonable cost and size
5. Capacity for testing viruses, bacteria, fungi and protozoa.

Most of these attributes have been considered in the range of systems suggested in this report for estuarine, freshwater, and terrestrial conditions.

Qualifications of and Limitations on Systems Described Herein

The enclosed systems described in this report are presented as suggested systems in which to attempt to develop methods for examining possible ecosystem and population level effects and impacts of MPCA's. These systems have not been validated yet in terms of efficacy with either

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[1]Containability as used in this report means the reasonable containment necessary to prevent release of the microorganisms under study to the work place (laboratory) or larger environment.

natural or genetically engineered MPCA's. They are presented here as a few of many possible systems that may be adapted for testing MPCA's for possible environmental impacts.

A final caveat is in order for those who may attempt to use these suggested, described systems for use with MPCA's without due adaptation: most of these suggested systems were developed to study the effects of chemical toxicants; therefore the different nature and modes of actions of MPCA's and chemicals must be taken into consideration if users of these systems are going to successfully adapt them to MPCA testing and study (See Section IV for further discussion on this point).

Each of the system sections were authored by those listed on title pages, and should be considered the results of the authors' effort and expertise. Further, each section or system description stands on its own merits in that it represents the authors' expertise and viewpoints.

ESTUARINE SYSTEMS

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PREAMBLE TO

MARINE AND ESTUARINE SYSTEMS

Three types of marine microcosms were identified to establish the potential environmental risks of genetically engineered and indigenous microorganisms. Specifically, simple, gnotobiotic and simulative systems (see table) vary from simple, two or more species systems to complex, interacting natural assemblages, respectively. The general underlying assumption was that the greater the physical and biological complexity of the experimental system, the greater the likelihood of observing ecological effects and ultimate fate of the test microorganism. In addition, the ability to relate ecosystem effects to natural marine environments was assumed to be directly related to the complexity of environmental simulation. The converse is probably valid for determining specific effects in individual, nontarget organisms, i.e., infectivity, pathogenicity, and toxicity endpoints are more optimally dealt with in simpler, single species tests.

In this section, a simple and two simulative systems are described; to the best of our knowledge, appropriate gnotobiotic systems in the marine area have not been developed. The simple system does not attempt to simulate any marine environment, but rather contains selected, trophically important species to which the test microorganism can be exposed through the surrounding seawater medium. The two simulative systems, the seagrass microcosm and the coastal microcosm, relate to shallow intertidal and deeper water marine environments, respectively.

The level of containment of the test microorganism will be dependent upon whether the microbe is natural, i.e., derived originally in the

natural environment or genetically engineered, i.e., an organism altered or manipulated anthropogenically and, as a result, not found in natural environments. If the former, then the containment procedures will not be as stringent as those for the latter. Each system has either explicitly or implicitly addressed the degree of containment relative to the type of microorganism tested.

General Characteristics and Properties of "Microcosms"

or Multispecies Test Systems Currently in Use

"Microcosm" Type	Components	Configuration of Components	External Exchange Conditions
imple	≥ 2 species; water, food, or air only as exposure medium; artificial medium (e.g. filtered or nutrient enriched water).	Direct or indirect species interaction via medium (e.g., air or water, etc.). Simulation conditions investigator dependent (e.g., water turbulence, air flow rates, etc.).	Usually static; completely contained
notobiotic	≥ 2 trophically related species chosen by investigator; sometimes water and soils/sediments artificial media (e.g. nutrient enriched water screened and/or sterilized soils, etc.).	All species and media capable of direct and indirect interactions; simulation conditions investigator dependent.	Static or manipulated flow-through (e.g. nutrient additions, artificial predation, etc.).
imulative	>> 2 species; natural intact biotic assemblages taken from field; natural and unmanipulated media.	All species and media capable of direct and indirect interactions, simulation conditions dependent upon natural system being simulated.	Unmanipulated flow-through or static based upon turnover time of natural system being simulated as situation requires.

A Simple, Enclosed, Multispecies System for the Evaluation of Infectivity, Toxicity, and Pathogenesis of MPCAs in Nontarget Aquatic Species

Office of Pesticides
Programs
ORD
USEPA

by

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1. SCOPE.

1.1 The application of pathogenic microorganisms for the control of pests has brought worldwide attention to the importance of these agents as microbial pesticides in their natural insect hosts. With continued efforts of geneticists, microbiologists, and insect pathologists, improved and more effective microbial pest control agents (MPCA's) will be available to potential producers of commercial MPCAs in the future. (Margalit et al., 1985). The possible intensive application of natural MPCAs and the introduction of genetically altered organisms into the environment presents a question of safety with regard to nontarget species. Because of the present use and the probable increase in registration, production and application of these agents in the future, the USEPA was given the responsibility for providing potential producers of MPCAs with guidelines for tests to determine the safety of MPCAs in nontarget species. (Couch et al., 1985).

1.2 To provide these methods, we are concerned with the design, development, and laboratory testing of a simple, enclosed aquatic system that can maintain nontarget species from at least three different phyletic groups for a minimum of 30 days for agent safety tests. We consider this a system to initiate preliminary studies on the potential impact of MPCAs on nontarget species. Our system does not attempt to simulate natural environments but rather to provide multispecies testing with limited interaction capability for evaluating endpoints of infectivity, pathogenicity and/or toxicity.

1.3 Other systems presented and discussed at this workshop will provide a larger opportunity for closer simulation of natural environments

and for studies of more complex interactions among microbial agents and nontarget species.

1.4 Preliminary studies indicate that three to four different nontarget species can be successfully maintained in the same system for a 30 day period. The design of this test system enables us to expose simultaneously a greater number of animals to MPCAs, and to provide some species interaction, thus expediting evaluation of the risks of these agents in nontarget aquatic hosts.

2. SELECTION OF NONTARGET SPECIES

2.1 Species from three representative estuarine phyletic groups were selected for testing:

1.) a fish, *Cyprinodon variegatus*; 2.) a shrimp, *Palemonetes pugio*, and 3.) two molluscs, *Rangia cuneata* and *Crassostrea virginica*. These animals were chosen because of their availability, laboratory adaptability, widespread estuarine and geographical distribution, and ecological role in the aquatic environment. (Couch, 1983). All 4 of these species can be readily found and collected in most typical estuaries along the entire Atlantic and Gulf coasts of the United States. Inland laboratories wishing to use these estuarine species as test animals, but unable to make collections themselves, can often obtain them from commercial sources.

2.1.1 The fish, *Cyprinodon variegatus*, inhabits shallow brackish areas of pools (small, sandy-bottomed sites in bays, coves, bayous, creeks, tidal cuts or channels) that are left isolated during low tides. They are tolerant of a wide range of salinities (0-142ppt) and temperatures (2-39°C) (Courtney & Couch, 1984). Collection using either seine net or bait trap can be easily made during

warmer months throughout the range, and anytime during the year in warmer latitudes. The grass shrimp, Palemonetes pugio, can be found in beds of submerged vegetation in shallow estuarine waters (Williams, 1984). They are normally most abundant in a salinity range of 10-20ppt, however this grass shrimp can be successfully maintained in lower or higher salinity range under laboratory conditions. During the warmer months they can easily be collected using a push net or fine seine net. The most favorable habitat for the clam, Rangia cuneata, is a combination of low salinity, high turbidity, and a substrate of sand, mud and vegetation. The common rangia is usually found in areas where the salinity seldom exceeds 18ppt and can be successfully maintained at lower or higher salinities. (LaSalle et al., 1985). This clam can be collected by hand or with tongs in shallow waters. The oyster, Crassostrea virginica, usually can be found growing on a variety of substrates in shallow water, halfway between high and low tide levels. The range of salinities favorable for C. virginica falls within two zones, the polyhaline from 18-30ppt and the mesohaline, from 5-18ppt. This oyster is tolerant of low salinities, but its survival is dependent on the range of fluctuations and the suddenness of the changes (Galtsoff, 1964). Oysters can be collected anytime of the year by hand or by the use of tongs.

2.1.2 Animals we tested were collected from the estuarine system located in the Pensacola/Gulf Breeze, Florida area. Adult C. variegatus and P. pugio were collected from Range Point, Santa Rosa Sound, by seine and push net; R. cuneata (2.5-4.5cm in size) were collected by hand in Escambia Bay from the shallow water near the mouth of

the Escambia River. Substrate consisted of a sandy-clay bottom intermittently covered with Thalassia beds. The clams were found partially or completely buried in the sediment. Small C. virginica (4-6cm in size) were collected from seawater troughs at the EPA Laboratory on Sabine Island.

3. TEST SYSTEM AND METHODS

3.1 Two 76 liter glass aquaria were filled to a 3 cm depth with clean crushed coral shell over a recirculating filtration system. Seawater from Santa Rosa Sound (25 ppt salinity) was added to these tanks and diluted to 15ppt with deionized water. Two shrimp were placed in each of 24 holding cups. Each cup consisted of a fine mesh screen cylinder cemented to a glass Petri dish bottom. Twelve shrimp holding cups were placed in a suspended cup tray in each tank with 20 fish, 16 clams, and 10 oysters, (See fig. 1) and were allowed to acclimate for 1 week. Animals that died before the maintenance observation period (30 days) began were replaced.

3.2 Two types of food, a commercial flake or live artemia, were used for feeding. In one tank the shrimp were fed one pressed flake-food pellet (approximately 0.056g/pellet) per holding cup. Approximately 0.5 g of unprocessed flake food was added to the open tank space for the fish. Shrimp in the second tank received approximately 10 ml of a suspension of live artemia per holding cup and 300 ml of live artemia suspension was added to the fish tank. It was noted that a greater amount of algae grew in the flake food tank, compared to the live Artemia tank. Animals were fed 3 times a week for 30 days. Oysters and clams probably filter-feed upon bacteria, some

algae and/or detritus available in the animals environment.

3.3 This test system can be adapted in several ways. To accommodate and maintain a greater or lesser number of animals, the size and shape of the test chamber can be varied. Any desired salinity can be maintained by either diluting existing seawater or by mixing commercially available sea salts with fresh water. Temperatures can be easily controlled by using common aquarium heaters or coolers.

4. PRELIMINARY EFFICACY OF SYSTEM AS MAINTAINED FOR 30 DAYS

4.1 The recirculating, static multispecies system maintained for 30 days proved to be very successful. Apparently, either method of feeding (flake vs. Artemia) can be used when applicable. The fish and clams had 100% survival with both feeding methods. A 99% survival rate was noted for the oysters in both feeding systems. Eighty percent(80%) of the shrimp that were fed live artemia and 84% of the shrimp fed flake food pellets survived. The higher mortality for the shrimp, compared to the other species, is within acceptable limits for this system. (Couch et al. 1983). This mortality can be explained by the shrimps' direct contact with one another in the holding cups, and their cannibalistic and antagonistic tendencies. A sampling of animals for each species group were examined grossly and appeared to be healthy.

Interaction among species in the system included direct ingestion of oyster fecal strings by fish, filtering of fecal materials from fish by oysters and clams, and exposure to fecal material from grass shrimp for all other species. This provides desirable interaction among test species because MPCA material may thus be exposed to potential natural degrading or enhancing factors.

5. RECOMMENDATIONS FOR USE OF SYSTEM WITH MPCA's

5.1 Two methods of exposing animals to MPCAs using this system are possible. Dosing thru feeding appears to be an efficient, natural, and certain method for ensuring internal exposure to certain MPCAs such as viruses, bacteria, and protozoans. (Couch, 1983). Fungi, such as *Lagenidium giganteum*, which attach externally to the cuticle of their hosts, could be introduced directly as zoospores into the water in test system in known concentrations.

5.2 Tissues from the exposed test animals can be examined, using specific histopathological, ultrastructual, serological, and genetic methods, to evaluate possible effects associated with infectivity, pathogencity, and toxicity.

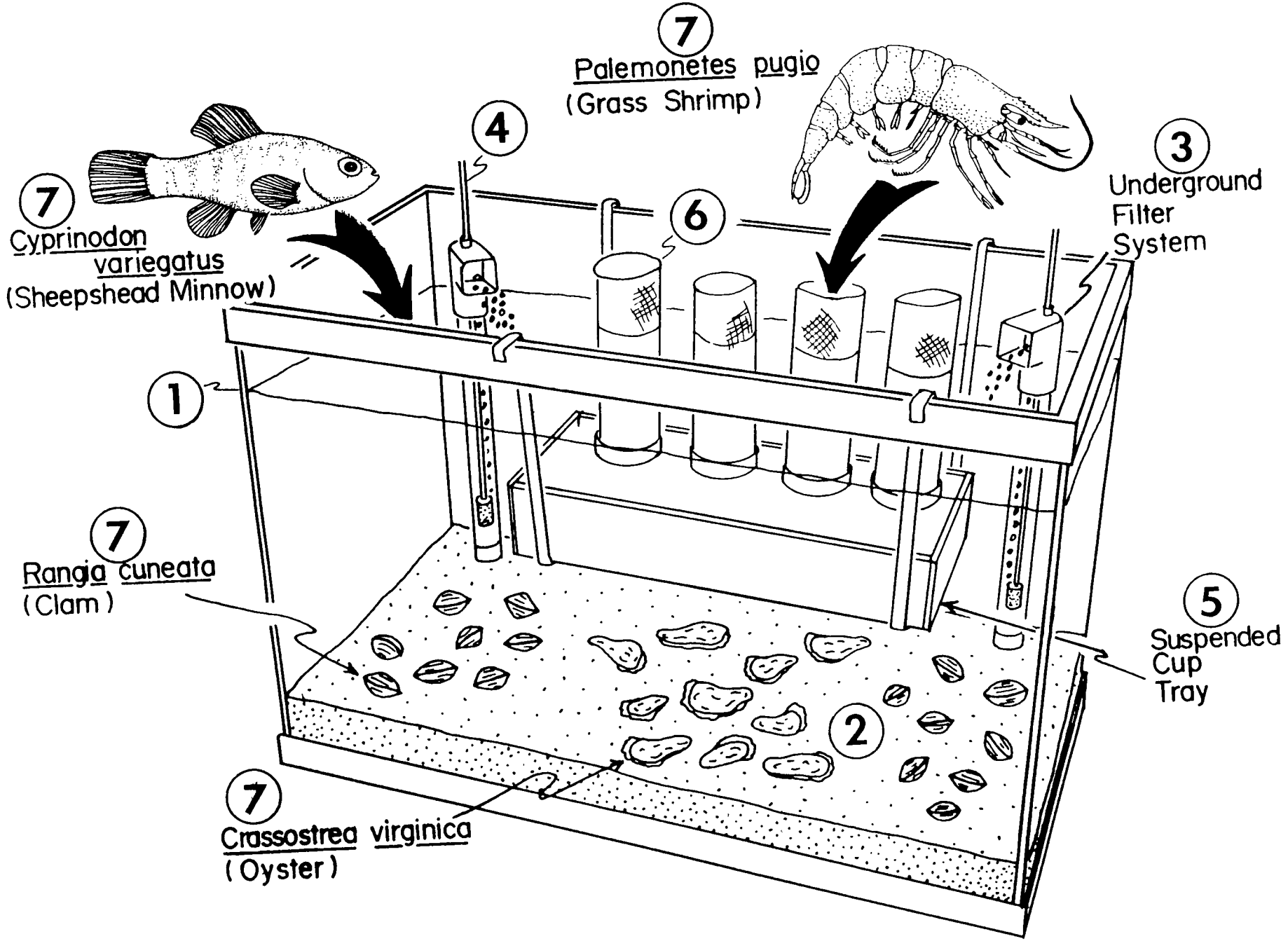
7
Palemonetes pugio
(Grass Shrimp)
4
3
Underground
Filter
System
7
Cyprinodon
variegatus
(Sheepshead Minnow)
6
1
7
Rangia cuneata
(Clam)
5
Suspended
Cup
Tray
2
7
Crassostrea virginica
(Oyster)

TABLE I COMPONENTS OF A SIMPLE MULTISPECIES (3-4 SPECIES) LABORATORY TEST SYSTEM FOR MPCAs
(COMPARE COMPONENTS IN THIS TABLE TO ITEMS IN FIGURE 1)

COMPONENTS	FIG* REF.	DESCRIPTION	SOURCE
TEST TANK AQUARIUM	1	ANY SIZE GLASS AQUARIUM DEPENDING ON DESIRED SYSTEM	COMMERCIALLY AVAILABLE FROM AQUARIUM SUPPLY OR CAN BE CONSTRUCTED
SEAWATER	-	0 - 33% SALINITY	COMMERCIALLY AVAILABLE SEA SALTS OR DILUTION OF SEAWATER
CRUSHED CORAL GRAVEL	2	COARSE GRAVEL SUBSTRATE COVERING THE UNDERGRAVEL FILTER SYSTEM	COMMERCIALLY AVAILABLE FROM AQUARIUM SUPPLY
RECIRCULATING UNDER-GRAVEL FILTER SYSTEM	3	TRAPS DIRT AND DEBRIS IN SUBSTRATE ALSO CREATES A WATER-AIR MIXTURE	COMMERCIALLY AVAILABLE FROM AQUARIUM SUPPLY
AIR SUPPLY	4	CENTRAL AIR COMPRESSOR OR PORTABLE ELECTRIC AIR PUMPS	COMMERCIALLY AVAILABLE FROM AQUARIUM SUPPLY
GLASS TRAY	5	A REMOVABLE GLASS TRAY WITH SHORT SIDES SUSPENDED IN THE TEST AQUARIUM	CONSTRUCTED USING COMMERCIALLY AVAILABLE MATERIALS
SHRIMP HOLDING CUPS	6	FINE MESH SCREEN CYLINDER CEMENTED TO GLASS PETRI DISH BOTTOMS	CONSTRUCTED USING COMMERCIALLY AVAILABLE MATERIALS
NONTARGET TEST ANIMALS	7	FISH, SHRIMP, CLAMS AND OYSTERS. OTHERS MAY BE SUITABLE.	COLLECTION FROM ESTUARIES OR OBTAINED FROM COMMERCIAL SOURCES
FOOD	-	FISH FLAKE FOOD BRINE SHRIMP (ARTEMIA)	COMMERCIALLY AVAILABLE FROM AQUARIUM SUPPLY
MPCAs TO BE TESTED	-	(MICROORGANISMS) VIRUSES, BACTERIA, PROTOZOA AND FUNGI	

*SEE FIGURE OF TEST SYSTEM FOR PICTORIAL DESCRIPTION OF COMPONENT

References

Couch, J.A., S.S. Foss and L. Courtney, 1985. Progress Report on Evaluation for Risks of an Insect Virus, Bacterium, and Protozoon to a Nontarget, Estuarine Crustacean. Office of Pesticides Program, ORD, USEPA, 2 p.

Couch, J.A., S.M. Martin, G. Tompkins, and J. Kinney. 1983. A Single system for the Preliminary Evaluation of Infectivity and Pathogenesis of Insect Virus in a Nontarget Estuarine Shrimp. Journal of Invert. Pathology 43, 351-357.

Courtney, L.A. and J. Couch, 1984. Usefullness of Cyprinodon variegatus and Fundulus grandis in Carcinogenicity Testing: Advantages and Special Problems. Natl. Cancer. Inst. Monogram 65:83-96.

Galtsoff, P.S. 1964. The American Oyster. Fishery Bulletin, Vol 64. United States Dept. of the Interior, Fish and Wildlife Service, Bureau of Commercial Fisheries. pp 4-5, 405-406.

LaSalle, M.W. and A.A. de la Cruz. 1985 Species Profiles: Life Histories and Environmental Requirements of Coastal Fishes and Invertebrates (Gulf of Mexico) Common Rangia. U.S. Fish Wildl. Serv. Biol. Rep 82 (11.31). U.S. Army Corps of Engineers, TR EL-82-4. 16 pp.

Margalit, J. and D. Dean. 1985. The Story of Bacillus thuringiensis var. israelensis. J. Am. Mosq. Control Assoc. Vol 1, No 1 p5

Williams, A.B. 1984. Shrimps, Lobsters, and Crabs of the Atlantic Coast of the Eastern United States, Maine to Florida. Smithsonian Institution Press. pp 76-78.

A Microcosm System for Evaluating System-Level Infectivity and Effects Of Microbial Pest Control Agents on Near-Shore, Non-Target Communities

For Office of Pesticides

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INTRODUCTION

Several commercial chemical companies are developing microbial pest control agents for application to agricultural crops to control specific pests. Under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), they are required to submit information on the potential impact of these agents on the environment. Since these agents cannot normally be "field-tested" to determine their impact on non-target organisms, laboratory tests can be extremely useful in evaluating potential impact.

The near-shore marine area could receive these agents by direct application for control of noxious insects or by transport of the agents from adjoining freshwater and terrestrial environments. A method is needed to test sensitive near-shore organisms in a holistic manner without an actual release in the environment. The procedure described herein captures some of the interactive complexities that characterize the structure and functions of the near-shore environment.

This test procedure orginally was developed to evaluate the effects of complex mixtures of chemicals on a rooted aquatic plant (seagrass) community (Morton, et al., 1986). Microbial pest control agents have not yet been tested in the system. Also, larger predators were excluded from the microcosms and this could result in some difficulties when extrapolating results to field situations. Nevertheless, the proposed microcosm test systems appears to be a reasonable tool for determining the potential impact of these agents on the near-shore environment.

NEAR-SHORE TEST PROTOCOL

1. SCOPE and PURPOSE

1.1 This test is designed to develop data on the impact of microbial pest control agents (MPCA) on the near-shore environment using a seagrass community as a sensitive test system. Effects of the MPCA on the microcosms can be determined through measurements of productivity; numbers, species richness and biomass of macroinvertebrates; and degradation rates.

2. MICROCOSM and SUPPORT FACILITIES

2.1 The microcosm is designed to simulate desirable physical, chemical and biological conditions existing in the natural environment where seagrass samples are taken. The support facilities (room, light fixtures, air system) ensure proper environment for testing. The experimental microcosms provide a community testing chamber that reflects conditions in the environment as much as possible.

2.2 The microcosms are plexiglass cylinders, 16.0 cm inside diameter, 50 cm high that are attached to a plate on the bottom. Cores collected from the seagrass beds are slipped intact from the core cylinder into the test cylinder.

2.3 Samples of the seagrass community are taken by divers with plexiglass cylinders 14.0 cm inside diameter and 50 cm high. The cores penetrate 10 cm deep in the grass beds and upon withdrawal, contain sediment with attendent macroinvertebrates and grass.

2.4 In the laboratory, microcosms can be operated with continuous flow of seawater or by replenishing the water in the microcosms daily.

(a) Flowing Water System

Unfiltered seawater of known quality is pumped into a settling reservoir above the test apparatus (Figure 1 (modified from Morton et al., 1986)). A 1-mm Nitex® filter is fitted over the delivery standpipe in the settling reservoir to exclude larger material from reaching and clogging the delivery tubes to the microcosms. Seawater is flowed from the settling reservoir to a baffled 20-liter Plexiglas® primary head-box suspended over the microcosms. Twelve glass standpipes, six at each end of the head-box, are fitted with silicone stoppers and calibrated to deliver approximately 200 mL/min. A larger diameter glass overflow standpipe at each end of the primary head-box maintains the water at a constant level. From the twelve standpipes, the water enters glass mixing tubes that deliver the seawater to twelve secondary headboxes. From each secondary head-box, seawater is delivered separately to four microcosms at a rate of about 40 mL/min. A glass cover is placed over the open end of the microcosm cylinder.

(b) Static (Renewal) System

In this system, 8 liters of seawater are placed in the microcosms. The water is drained (syphoned) from each microcosm every 24 hours and replaced by fresh seawater of known salinity, pH, temperature and dissolved oxygen.

2.5 Decomposition

Leaf litter or detritus bags can be added to each microcosm in order to determine impact of the microbiological pest control agents on decomposers. (Weight loss of bags in

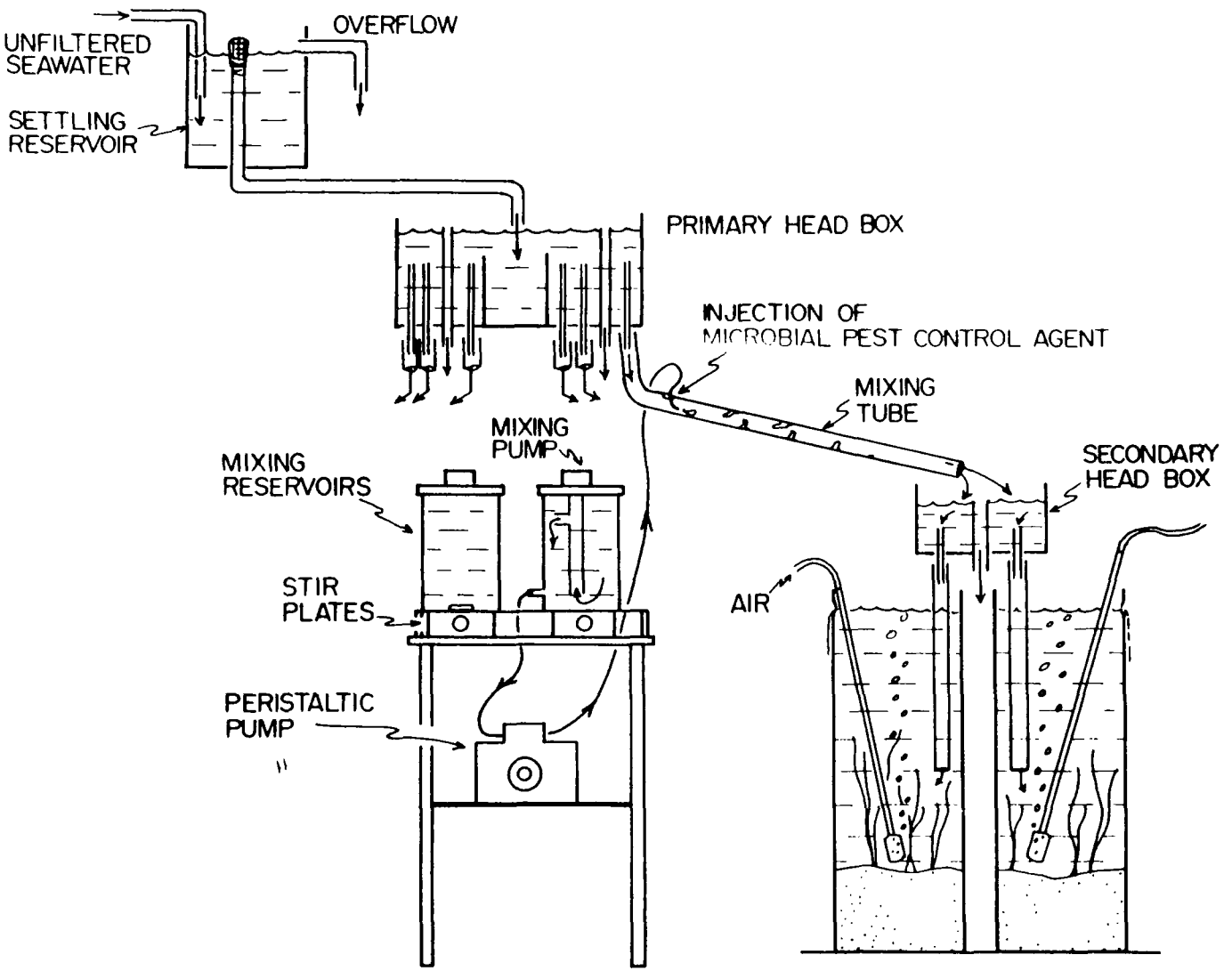
UNFILTERED SEAWATER
OVERFLOW
SETTLING RESERVOIR
PRIMARY HEAD BOX
INJECTION OF MICROBIAL PEST CONTROL AGENT
MIXING TUBE
MIXING PUMP
MIXING RESERVOIRS
SECONDARY HEAD BOX
STIR PLATES
AIR
PERISTALTIC PUMP

FIG. 1

treated microcosm is compared with that in control - weight loss is equated to degradation). Bags are constructed of 2 mm mesh Nytex nylon netting and 10 cm square. Glass rods anchor the bags in the microcosms just above the sediment.

2.6 Lighting

Lighting for the test is provided by four 400-WATT multivapor lamps and four high-intensity fluorescent bulbs rated at 250 watts. Suspended 46.0 cm above the microcosms, the bulbs provided an average light energy of 217 $\mu E/m^2/S$. Lights are connected to a 24 hour timer (12h light and 12h dark).

2.7 A summary of components of the microcosms is found in Table 1.

3. DECONTAMINATION

3.1 Tests must be conducted to determine the methods and materials required to decontaminate microcosm glass tubing, or other materials that may contain the test organisms. Appropriate disposal procedures must be employed if decontaminating agent is classified as a hazardous material. Care must be taken to collect and decontaminate flow-through or renewal water.

4. Definitive Test

4.1 The flow chart for conducting the test is found in Table 2. (Modification of Morton et al., 1986). There should be at least 12 replicates of exposed microcosm in each test. The test is conducted for six weeks with flowing seawater and for two weeks with replenished water.

4.2 Harvest and Treatment of Samples.

4.2.1 The 48-microcosm test system is harvested randomly at the end of

the test period. Fifteen seagrass leaves with associated epiphytes are taken from each treatment for analysis of biomass and chlorophyll content. Remaining contents are sieved to separate plant and sediment portions and placed into 0.5 mm nylon bags. All portions should be immersed in propylene phenoxetol in seawater to relax organisms. Plant materials are fixed in five percent formalin until the animals are picked from them and grass weights determined. Sediment portions are placed in ten percent formalin in seawater with 100 mg/liter rose bengal stain. Sediments are rinsed with freshwater, then sieved through 1.0 and 0.5-mm sieves. Sieved material is stored in 60 percent 2-propanol until the macroinvertebrates can be sorted and identified. All macrofauna are identified to the lowest taxonomic level possible.

4.2.2 Litter bags can be removed at intervals for measurements of rates of decomposition or can be removed at the end of the test. Bags are removed from the microcosms, rinsed in freshwater, dried at 100° C for 24 h, weighed, and the weight change (loss) calculated.

4.2.3 Measurements of the Thalassia leaf samples includes chlorophyll a per g tissue or per cm^2 blade area. Effects on epiphytic communities are observed by measuring differences in epiphytic biomass (ash free dry weight-AFDW) per cm^2 of Thalassia leaf as a biomass indicator, chlorophyll a per g AFDW epiphyte tissue as a community health indicator, and chlorophyll a per cm^2 leaf area as an indicator of photosynthetic potential. Details of these analyses to determine the health and productivity of the epiphytes can be found in Price, et al. (19).

4.2.4 A summary of criteria for effects is presented in Table 3.

Table 1. Descriptive Table - Seagrass Microcosm

Component	Description	Source	Species
Seagrass	Seagrass beds cored to a depth of approximately 10.0 cm by Plexiglas cylinder (14.0 cm inside diameter, 50.0 cm height) grass, sediment and attendant macroinvertebrates are sampled.	Within euphotic zone of near-shore environment, particularily temperate sub-tropic, and tropical waters.	*Thalassia testudinum* used in this protocol, however, other grasses such as *Zostera marina* may be employed.
Macrobenthos	Taken as part of seagrass core. Larger macroinvertebrates eliminated from microcosms.	Taken in same location as grasses.	The kinds and numbers of species vary with season and water quality at time of sampling. Examples of macroinvertebrates collected in the fall in Santa Rosa Sound, FL, are as follows: Annelida *Aricidae philbinae* *Armandia maculata* *Fabriciola spp.* *Mediomastus californiensis* *Nereis pelagica* *Pista* sp. A *Prionospio heterobranchia* *Tharyx marioni* Arthropoda *Elasmopus laevis* Mollusca *Bittium varium*
Leaf Litter Detritus Bags	Bags of 2 mm nylon netting, 10 cm square. Either dried or fresh seagrass are added to bags that are anchored in the microcosm by glass rods.	Leaves for bags taken from same grass beds as seagrass cores.	The leaves in the bags should be the same as those used in the microcosms.

Table 1. (Cont'd)

Component	Description	Source	Species
Microcosms	Consist of Plexiglas cylinders slightly larger than coring apparatus (16 cm inside diameter; 50 cm high). Seagrass cores slipped inside microcosm so each one contained intact core of seagrass and attendent sediment, macroinvertebrates, and epiphytes. Experimental apparatus shown in Figure 1.	Plexiglas tubes can be purchased at most local plastic pipe supply companies or plumbing supply companies.	
Lights	A combination of 4 400-watt multi vapor lamps (Model Mur 400 U) and 8 215-W cool-white fluorescent lamps (Model E96 T12/CW/1511). Lights are connected to 24 hour timer (12h light and 12h dark). An average of 217 $\mu E/cm^2/S$ were delivered to the surface of the microcosms.	Electrical Supply Co.	

Table 2.

FLOW CHART

Collect Cores From Field

Establish Microcosm in Laboratory

Begin Daily Measurements of pH, Temperature, Salinity

Acclimate to Laboratory Conditions

Add Microbial Test Agents

Expose for Six Weeks
(Daily Water Renewal)

or

Expose for Two Weeks
(Static Test)

Harvest Microcosms

Analyze

Macroinvertebrates:
- Number
- Kinds
- Species Diversity
- Patterns of recognition

Rate of Decomposition (Weight Loss of Litter Bags)

Chlorophyll Content and Growth of Seagrass

Kinds and Density of selected Microbes

Table 3.

Criteria for Effects - Seagrass Microcosm

Component	Criteria	Method	Reference
Seagrass	Chlorophyll a per gram blade area	Acetone extraction Spectrophotometer analysis	Strickland and Parsons (1972)
	Growth rate	Movement of aluminum tape during experiment	Modification of Zieman (1974)
Macroinvertebrates	Number of individuals per species Species richness Total number of individuals Species diversity Cluster or discriminate analysis or other methods of pattern recognition	Microcosms are harvested at the end of desired time. Macroinvertebrates are seived, (1.0 and 0.5 mm), identified and enumerated. 10 numerically dominant species can be analyzed.	Morton et al. (1986)
Microbes	Enumeration of desired functional groups (Nitrification, Sulfur, etc.)	Plate counts	

References

Morton, D.R., Duke, T.W. Macauley, J.M., Clark, J.R., Price W.A., Hendricks, S.J., Owsley-Montgomery, S.L., and Plaia, G.R. In Press. "Impact of Drilling Fluids on Seagrasses: An Experimental Community Approach." Proceedings of a Symposium entitled "Community Toxicity Testing." 1986, 25 pp.

Price, W.A., Macauley, J.M., and Clark, J.R. In Press "Effects of Drilling Fluids on Thalassia testudinum and Its Epiphytic Algae." 1986, 21 pp.

Strickland, J.D.H. and Parsons T.R. (1972). A Practical Handbook of Seawater Analysis. Bulletin 167 (2nd ed). Fish. Res. Bd. Can. 310 p.

Zieman, J.C. (1974). Methods for the study of the growth and production of turtle grass, Thalassia testudinum Konig. Aquaculture 4, 139-143.

EXPERIMENTAL MARINE MICROCOSM TEST PROTOCOL
AND
SUPPORT DOCUMENT

Measurement of the Ecological Effects, Fate and Transport of Living Microorganisms in in a Site-Specific Marine Ecosystem

by

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PREFACE

Commercial chemical companies are required, under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) to submit information about the agricultural use of indigenous and genetically engineered organisms. This information is used by the Environmental Protection Agency to develop an environmental and/or risk assessment for each test organism prior to the approval of a production permit. This assessment, in the marine environment, would include effects of the organism on the receiving body or ecosystem and the potential for indirect human exposures through the consumption of marine organisms.

The microcosm test protocol described herein possesses many of the physiochemical and biological complexities of natural coastal marine systems. This was achieved by coupling natural and undisturbed assemblages of the water column and benthic communities in a realistic manner. This test system, therefore, will provide a diversity of microenvironments in which the fate and effects of the introduced test organism can be measured. If the test organism survives and proliferates in the microcosm, this protocol has the potential to estimate the specific impacts at the ecosystem level of organization. It may also provide estimates of the air and advective transport of the test organism to adjacent areas. Such information enables the user to develop a more accurate environmental risk assessment of the test organism as compared to those assessments founded upon test systems possesing lower levels of physical and biotic complexities. In addition, the recommended experimental procedure has some internal checks designed to establish the validity of the test results. Furthermore, the knowledge and control of inputs and outputs of variables, perturbations

and processes minimize the variance between replicate microcosms. This dramatically enhances the ability to detect differences between the exposed and control sets of microcosms utilizing standard statistical procedures.

ASSUMPTIONS AND CONSTRAINTS

The protocol described below is a modification of one designed to establish the fate and effects of toxic chemicals (Perez, 1983). Because of the limited information regarding such test procedures using living microorganisms, these modifications were made, primarily, on a conceptual basis. As a result, there are a number of assumptions and constraints associated with the use of this protocol.

First, we believe that number of replicates per treatment condition and the error rate found to be adequate for toxic chemicals testing, will also be adequate for detecting differences between control and exposed treatments when used for microorganism testing.

Second, we assume that the ecological effects and fate of the test organism in the laboratory microcosms will be sufficiently similar to that realized in a field system when exposed to the test organism. However, until field validation studies are performed, environmental risk assessments, based on data derived from this protocol, will be constrained by this assumption.

The last constraint is that natural sunlight is not simulated in this protocol. If the test organism (1) accumulates in the surface microlayer (i.e., the interface between the water and the overlying gas phase) and (2) is sensitive to ultraviolet radiation, this protocol will potentially overestimate the survivability and proliferation of the test organism in the marine environment and thus provide a worst case scenario.

Finally, some words of caution in the application of this test protocol are necessary. The data and ensuing assessments derived from this procedure are constrained in two primary areas. (1) Because of the size of the microcosm tank, macrofauna such as fishes and large crustaceans have been excluded. As a result, some economically important species cannot be tested in these systems. In addition, macrofauna exclusion could possibly affect the responses of some of the measured ecological effects. Whether macrofauna significantly influence such variables (phytoplankton, zooplankton, nutrients, chemical distribution, etc.) for different ecological systems, particularly within the experimental time frame of 30 days, remains to be established. (2) The microcosm test system was developed from a temperate Northeast (U.S.) estuarine ecosystem where intertidal and reef (e.g., oyster, coral, etc.) subsytems are either small in area or missing. The applicability of this protocol to other site-specific marine ecosystems where either shallow conditions and/or major reef subsystems exist has yet to be determined.

CONTAINMENT

This protocol is written and the system designed to minimize environmental release and human exposure to any Genetically Engineered Material (GEM). The level of containment designed, in our view, exceeds, or at least equals, the National Institutes of Health, Class III biological safety level (Anon.,1984). If the test organism is an indigenous microorganism, that is, one naturally found in field environments, containment conditions can be relaxed to the degree that the primary concern will be to prevent the contamination of the experimental control tanks.

TEST PROTOCOL

1. SCOPE AND PURPOSE

1.1 The standards of this subsection are designed to develop data on the fate and ecological effects of living microorganisms on marine ecosystems. The United States Environmental Protection Agency (EPA) will use these data to assess the hazards these organisms may present to the marine environment and potentially. human health, through uptake and accumulation in human food species.

2. DEFINITION OF A NATURAL SYSTEM

2.1 The natural marine system (designated as "natural system") at a particular geographic location consists of a coupled water column and benthic subsystem. Within both the water column and benthic subsystems, certain physical and chemical conditions, and biological properties exist. These conditions and properties are simulated in laboratory microcosms as described in Section 4.2 and 5.6.2.

2.1.1 The physical and chemical conditions of the natural system are defined in terms of its:

(1) Boundaries - The boundaries of the natural system are delineated by the maximum excursion of water above and below a selected point during one complete tidal cycle.

(2) Light - The light regime of the natural system is characterized by the photoperiod, the best estimate of average monthly incident radiation (total incident radiation per day divided by photoperiod), the average seasonal water extinction coefficient, the average daytime vertical light intensity for the

entire water column, and the depth at which the average light intensity occurs.

(3) Sea water - That fluid existing within the boundaries of the natural system.

(4) Sea water turbulence - The average water motion realized for the entire water column of the natural system during the period of experimentation.

(5) Sea water turnover rate - The time required for one complete water turnover or exchange within the defined boundaries of the natural system.

(6) Ratio of benthic surface area to sea water volume - The ratio obtained by dividing the calculated benthic surface area of the natural system by the best estimate of water volume of the system.

(7) Sediment - The bottom substrate existing, at the mean water depth, within the boundaries of the natural system.

(8) Sea water flow rate over sediment surfaces - The average tidal velocity over the bottom as measured in the natural system.

2.2 Biological Conditions and Descriptions

2.2.1 The biota of the natural system is characterized by the organisms in the water column and benthic subsystem.

(1) The water column is characterized by the numbers and species composition of phytoplankton, zooplankton, and transient larval forms found in the sea water at the designated collection site at a mid-tide. The water column, with its assemblages of biota, is obtained from the natural system by the methods described for filling the microcosms (Section 5.6.2(1)).

(2) The benthic subsystem is characterized by the sediment type and the structural composition of the benthic community, i.e., numbers, species composition, and feeding types of organisms collected from the benthic boxes (Section 4.2.1 (2,3)) and retained on a 0.5 mm screen.

(3) If the natural system has more than one distinct benthic community, then those having direct routes to human consumption or which are of significant economic and aesthetic importance should be considered for experimental use.

3. TEST ORGANISM

3.1 Information Required

3.1.1 A detailed description of the test organism including levels at which human health may be affected, and any known methods for identifying and measuring the organism must be supplied. All known information regarding the decontamination of air, water, and surfaces contaminated with the test organism should be included.

3.1.2 The degree of purity and the identification and quantification of any contaminating organisms, if known, should be determined.

3.1.3 It is extremely important to have a knowledge of, or a best estimate of the test organism's mode and form of entry into the marine environment. Specifically, does the test organism enter by atmospheric or aqueous sources? Is the test organism in association with other materials such as a dispersing agent? As will be discussed in Section 5.5.1, the extent of such information will determine the method by which the test organism is added to the experimental microcosms.

3.2 Methods must be described for collecting and measuring the test organism in and/or on the air, sea water, surface microlayer, sediment, biota (both whole animal and specific tissues if necessary), and microcosm surfaces.

4. MICROCOSM FACILITY

4.1 The microcosm facility (Figure 1) is designed to simulate relevant physical, chemical, and biological conditions existing in the natural system described in Section 2. It is composed of two basic parts: (1) the support equipment (room, waterbath, light and turbulence fixtures, test compartments, and an air evacuation system) and (2) the experimental microcosms (tanks, paddles, benthic cylinders, pumps and pump air supply). Two physically separate units (one each for exposed and control microcosms) must be used when biological substances are being tested.

4.1.1 Support Equipment (See Figure 1)

(1) All testing must be performed in a room that eliminates any light source other than that provided by the microcosm facility.

(2) The microcosm tanks must be held in a waterbath of sufficient dimensions, with sufficient heating/cooling capacities, as to provide uniform test temperatures within all treatment conditions and maintain those temperatures within 1 C of the natural system at the time of the experiment.

(3) A canopy above the waterbath should hold sufficient fluorescent lighting to provide an even light distribution at the desired light intensity over all the experimental microcosms. Light is provided by "Cool White" fluorescent bulbs, and the photoperiod controlled by a 24 hour timer. A plexiglass cover, the same length and width

of the canopy, is mounted directly below the fluorescent lamps.

(4) The open area between the canopy and waterbath is enclosed and sealed with plexiglass. The front plexiglass covers are removable to facilitate setting up and filling the microcosm tanks. These covers are equipped with rubber gloves and air locks (Figure 6) to facilitate the safe collection and removal of samples. The air locks will be designed to allow the application of an appropriate decontamination agent (5.5.5) to the exterior of sampling vials, pipets, etc. prior to removal from the chamber. The air locks are vented to the air exhaust system (Section 4.1.1[6]). Plexiglass panels are also mounted transversly within the waterbath/canopy module. These panels are joined and sealed to the horizontal plexiglass sheet mounted below the fluorescent lights and extend approximately ten cm. below the water surface, to provide airtight test compartments (Figure 6). The first three compartments will each hold two microcosm tanks. The fourth compartment will hold three tanks. All nine tanks within a given waterbath/canopy unit (Figure 1) will be reserved for one specific treatment condition (i.e., exposed or control (Section 5.6.1 (1)). The individual test units (exposed and control) must be physically separated from each other to reduce the chance of contamination of the control tanks. Access to the microcosm tanks during a test is via the rubber gloves and air locks mounted in the front of each test unit.

(5) Turbulence is provided by a system that consists of an electric motor, motor controller, chain drive, mounts and drive shafts. The motor should have the capability of maintaining speeds of 1 to 40 RPM. This system is mounted on the canopy with the drive shafts

extending through, directly above each microcosm tank (Figure 1). The paddles are bolted to the drive shafts.

(6) A slightly negative air pressure is maintained within each test compartment. An exhaust fan draws air from the test compartments through a PVC pipe manifold. The exhaust air is passed through HEPA particle filters capable of removing the test organism (Figure 1). The filtered air is vented through a stack outside of the laboratory building. The exhast stack should be as far removed as possible from the laboratory air intake.

4.2 Experimental Microcosms

4.2.1 The experimental microcosms are designed to provide a multi-trophic level experimental chamber that has coupled pelagic (water column) and benthic communities similar to those existing in the natural system.

(1) The microcosm tanks are 140 liter, cylindrical fiberglass containers (Figure 3). The tanks are held in position by a plywood "collar" mounted in the water bath trough and encircling each tank approximately 15 cm below the rim.

(2) The benthic boxes are thick walled PVC cylinders, open at both ends, and are approximately 30 cm long (Figure 5). The inner diameter of the benthic cylinder must be such that the microcosms sediment surface area-to-water volume ratio equals that of the natural system (Section 2.1.1(6)).

(3) The benthic cylinders are used by divers to collect undisturbed sediment cores from the prescribed portion of the natural system 2.1.1 (7) and 2.2.1 (3). The depths of the core should be equal to or greater than the depth of biological habitation or activity

but not exceed 25 cm. The bottoms of the cylinders (cores) are sealed, prior to installation in the microcosm tanks, with PVC plugs secured by PVC bolts (Figure 5). The cores, at this point, should be watertight i.e., the water overlying the sediment core will not flow downward between the sediment periphery and the cylinder. If shells and/or other debris, biologically derived tunnels, etc. preclude this condition, then such cores should be rejected. The length of time between collection of the cores and initiation of the test should not exceed two weeks. During the interim period the cores can be held in the laboratory, submerged in flowing, well aerated sea water.

(4) Waterflow over the sediment core in the microcosm is provided by an air displacement pump (Figures 3,4,5). The dimensions of the pump should be sufficient to provide a flow comparable to the average bottom water flow rate of the natural system. The pump is positioned in the tank adjacent to the benthic core (Figures 1, 3). The benthic core is mounted in the microcosm tank so that the cylinder overflow port is approximately 1 cm. above the tank water level. The cylinder and pump are supported from fiberglass angle brackets bolted to the inside of the tank (Figures 3,5). Water from the microcosm tank enters the pump through a small (2 cm) hole in bottom. When air pressure is applied to the pump, a silicon rubber check valve inside the pump is forced downward and seals the bottom hole. This forces water in the pump through a connecting tube and into the benthic cylinder. Water entering the cylinder is directed into several horizontal streams by a diffuser (Figure 5) in order to minimize sediment disturbance and re-

suspension. Air pressure to the pumps is controlled by an electronic timer operating a solenoid valve. The air continues to force water out of the pump for a preselected period of time. The pumping time must be less than that required to force all the water out of the pump. The timer then activates the solenoid valve which stops the air flow, allowing water to re-enter the pump from the microcosm tank. Again, after a pre-defined refill period (at least long enough to allow the pump to completely refill), the air resumes flowing and the cycle is repeated. Water overflows from the benthic cylinder back into the microcosm tank through a small hole located approximately 5 cm. above the sediment level. The rate of waterflow should approximate the mean tidal velocity measured at the sediment surface of the natural system (Section 2.1.1(8))

(5) The light intensity over the sediment surface should approximate that incident in the bottom meter of the natural system. This is accomplished by placing a light filter or cover over the top and sides of the benthic cylinder. See Section 5.5.4 for establishment of water column light intensity.

(6) Turbulence is generated by paddles that are connected to the drive shafts described in Section 4.1.1(5) and Figures (1,2). The paddle consists of a vertical shaft with two rows of smaller diameter shafts fused to it radiating horizontally outward (Figure 2). The length and number of arms are dependent on the microcosm tank, benthic cylinder and pump configurations, and the level of turbulence desired. The turbulence level in the microcosms is controlled by paddle configuration as well as the speed of rotation.

Once the paddle configuration is established, the speed of rotation is adjusted so that the dissolution rate (described as weight loss/time) of pure gypsum ($CaSO4$) blocks (1.5cm x 2.5 cm x 1.0 cm), distributed throughout a microcosm tank, is statistically equivalent to the dissolution rates of gypsum blocks measured in the water column (top to bottom) of the natural system during the season of the experiment. Dissolution rates should be measured and water turbulence adjusted prior to each experiment.

(7) Several small fiberglass disks, approximately 1 cm2, are suspended below the water surface. These disks should be constructed from the same material as the tank. They will be used to provide estimates of the amount of test organism adhering to the test tank.

(8) The tank is covered with a plexiglass cover. The cover serves to prevent the release of the test organism to the laboratory atmosphere and allows for the monitoring of the atmosphere above the water surface for aerosolization of the test organism. The cover is made of 0.25 inch thick plexiglass and is mounted, with an air tight seal, to the tank (Figure 3). The cover has a 1.5 inch hole in the center over which is mounted a ball bearing pillow block (flanged unit). A fiberglass rod, approximately 18 inches long, is inserted in the pillow block. The pillow block unit is seated on a silicon gasket and makes an air tight seal with the plexiglass cover. The fiberglass rod is attached, with couplings, to the paddle (lower end) and the drive shaft (upper end). Another hole is drilled near the edge of the cover and fitted with an air line coupler for the benthic pump (Figure 7).

Air line fittings (Figure 7) are also provided to allow air to be drawn across the water surface. The rate of air flow must be sufficient to maintain the concentrations dissolved gases eqivalent to field levels. Other openings can be made in the cover to accommodate additional sampling if necessary. Any openings in this cover must be equiped with plugs or covers which allow them to be sealed.

(9) All test equipment and sampling devices that come in contact with the sea water and test organisms must have been decontaminated by appropriate means (5.5.5), washed with detergent and thoroughly rinsed with deionized water prior to use.

5. TEST PROCEDURES

5.1 Test Purpose

5.1.1 The purpose of the experimental ecosystem (microcosm) test is to determine the fates and ecological effects of introduced microorganisms within a site specific marine or estuarine ecosystem within a 30 to 60 day period.

5.2 Assumptions

5.2.1 The ability to extrapolate the results derived from the experimental microcosms to the natural system is based on the correspondence of the biological events expressed in the experimental control tanks and those realized in the natural system at the time of the experiment. For those biological events that are statistically equivalent (a = 0.05), it is assumed that the results observed in the experimental tanks receiving the test organism would also be realized in the natural system, if similarly stressed.

5.2.2 It is assumed that the arbitrarily chosen mode and form of entry

of the test organisms into the microcosms will simulate the known or predicted entry into the natural system.

5.3 Test of Assumption 5.2.1

5.3.1 The fate and ecological effects of the test organism can be ascertained in the experimental microcosms and related to the expected perturbation behavior in a natural system provided the levels of all relevant independent variables, either simulated and/or realized in the microcosms, are equal to those in the natural system at the time of the test.

5.4 Use of Test Results

5.4.1 The data generated from these microcosms will be used to better understand the potential fate, transport and ecological effects of the test organism in marine ecosystems. The assessment should discuss the potential ecological effects and the possibility of indirect human exposures associated with the mode and form of release of the test organism in a specific marine ecosystem.

5.5 Preliminary Testing

5.5.1 Input of Test Organism: Mode, form and exposure levels. The manufacturers recommended mode (i.e., atmospheric, aqueous) and form (i.e., liquid, solid, with or without carrier) of application of the test organism into the natural environment will be simulated in the experimental microcosms.

5.5.2 Input of Test Organism: Desired exposure concentration. The nominal exposure concentration of the test organism within the microcosms should be at the manufacturers recommended application level.

5.5.3 Sampling and measurement. Procedures and techniques must be obtained or developed for sampling and measuring the test organism in sea water, air, surface microlayer, sediment, tissues, and microcosm surfaces prior to the definitive test.

5.5.4 Light Intensity. Preliminary tests should be performed (without the test organism) to establish the proper light intensity over the microcosms. These tests should be done with all the microcosm equipment and facilities (i.e., waterbath, tank, paddle, benthic box and pump) in place. The tests should be performed at several light levels, based upon conditions listed in Section 2.1.1 (2), with photoperiod equal to ambient conditions, for at least 14 days. Those light levels producing phytoplankton abundances that are statistically equivalent to the natural system should be used in the definitive test.

5.5.5 Decontamination: Tests must be performed to determine the methods and materials required to decontaminate microcosm test compartments, air locks, and sample containers that may contain the test organism. Appropriate disposal procedures must be employed if the decontaminating agent is classified as, or the investigator feels is, a hazardous material.

5.6 Definitive Test

5.6.1 The definitive test consists basically of (a) the initial setup and filling, (b) addition of the test organism, (c) daily sampling and water exchange, and (d) final sample collections.

(1) Experimental design: There should be at least nine replicate control and nine replicate exposed microcosms established for each experiment.

5.6.2 The microcosm test system is placed in operation as follows:

(1) The test water is collected from the natural system at mid-tide by hand bucketing or non-destructive pumping, (diaphragm pump) and transported to the test facility. If the natural system is vertically stratified in terms of temperature and/or salinity, the initial filling water should be a mixture (average) of water collected from various depths. Subsequent collections for water exchange (Section 5.6.4(4)) and biological sampling (Section 5.6.6) are performed in the same manner.

(2) During the filling process, the water is distributed equally among the microcosm tanks until the prescribed volume is reached.

(3) The benthic cylinders containing the sediment cores (Section 4.2.1(2,3)) are carefully placed in the tanks (Section 4.2.1(4)) so as to minimize sediment disturbance.

(4) The benthic pumps are placed adjacent and connected to the benthic cores (Figure 3). The plexiglass covers, with paddles, water sampling and exchange tubes attached (Figure 3), are placed on the tank. A short flexible tube is used to connect the benthic pump to the air line fitting on the under side of each cover. The covers are then bolted or otherwise clamped to the tank rims to provide an air tight seal. Air lines (Figure 3) supplying low pressure air to the benthic pumps are attached to the air line fitting on the top of the covers.

(5) The paddles are connected to the drive shafts. The speed of rotation is established (see Section 4.2.1(6)) to generate a turbulence level equivalent to that in the natural system.

(6) Light intensity at the microcosm tank water surface is

controlled by adjusting the shading of fluorescent lights with aluminum foil or similar material to achieve levels as determined in Section 5.5.4. Photoperiod is set equal to ambient conditions.

(7) Temperature in the water bath is adjusted to the average daily temperature of the natural system being simulated.

(8) After any disturbed sediment in the benthic cylinders settles, the low pressure air to the benthic pumps is turned on and water flow to the benthic cylinders begun at an average rate equal to the average tidal flow over the benthic substrate in the natural system. (Sect. 4.2.1(4)).

5.6.3 Addition of the test organism

(1) The test organism is added to the water column of the experimental microcosms in the mode, form, and concentration established in Section 5.5.1 and 5.5.2.

(2) If a carrier other than water is used to disperse the test organism in the natural system (Section 5.5.1), it should also be used to apply it in the test system. Analysis of effects will be based on the integrated effects of both the test organism and carrier.

(3) If input of the test organism to the natural system is other than a one dose application (i.e., multiple application, runoff), the test organism must be added to the microcosm tanks in the same manner as the initial dose whenever there is a water exchange (Section 5.6.4(3,4)) but only in quantities sufficient to achieve the desired test concentrations in the replacement water.

5.6.4 Performance of the test

(1) The experiment is run for 30 days. It is recommended that this

test be conducted during the temperature extremes of an annual cycle. Two tanks (both from the same compartment) from each treatment condition must be sacrificed and comprehensively sampled (at days 10, 20, and 30) in order to determine the fate dynamics of the test organism. If, after thirty days, the test organism in the water column has not reached steady state, the operation of the remaining six tanks (three each of control and exposed) will be continued for an additional thirty days.

(2) Water turnover volume and frequency is established to match the exchange rate of the natural system (Section 2.1.1(5)). Water exchange in the microcosm tanks should be scheduled at least three times a week and should coincide with the biological and chemical sample collections. The discreet volume of water removed during the water exchange equals the calculated amount minus that volume removed during biological and chemical sampling.

(3) Water removal from the microcosm tanks for water exchange, biological, and chemical sampling is accomplished by drawing the desired volume of tank water into the waste water vacuum flask. Each flask is equiped with a float switch (Figure 6) which will turn off the vacuum pump should the water volume in the flask exceed the desired volume. Samples of this water can then be taken for zooplankton enumeration. The remaining water can be drained into the waste water tank (Figure 1). The waste water tank will have had a sufficient volume of the disinfectant added (5.5.5) to kill the test organisms present in the waste water. This process is repeated for each experimental tank. The waste water must be tested to insure that the test organism is not

present before being disposed.

(4) On water exchange days, the refill water tank is filled with newly collected water (5.6.2 [1]). Phytoplankton and zooplankton samples are collected from the refill water for comparison with those taken from the control and exposed microcosm tanks. The refill tank is placed in the refill chamber and the chamber sealed. The water and vacuum lines are connected via the gloved ports. Water replacement is accomplished by first drawing the desired volume of newly bucketed water from the refill water tank into the refill water vacuum flask. This water is then allowed to drain into its respective experimental tank. After all the tanks have been refilled, the refill water tank is disconnected (via the gloved ports) from the refill line and the chamber and refill tank disinfected as a precaution in case of accidental or inadvertent contamination. The refill tank can then be removed, washed and readied for the next use.

(5) Open Petri dishes containing an appropriate growth medium can be placed at various locations within the test, replacement water, and waste water chambers during the test period. Any indication of the test organism growing in the dishes would be positive proof of contamination within the chamber. Negative results (i.e., no growth), however, would not necessarily be proof of no contamination.

5.6.5 Test Organism Fate Sampling

(1) Samples of the test organism in the air and water column should be taken on water exchange days prior to the actual replenishment. Samples from the surface microlayer, sediments,

benthic organisms and microcosm surfaces will be taken from those tanks sacrificed at day 10, 20, and 30 (5.6.4 [1]). All sampling and other microcosm operations (water exchange, etc.) must be performed using the gloved ports mounted in the front plexiglass covers. All samples must be placed into bottles, tightly sealed and decontaminated in the air lock before removal from the test chamber. Likewise, all sampling materials (pipettes, forceps, etc.) must be decontaminated before removal from the test chamber. The samples must be opened and analyzed at an appropriately equipped site remote from the test chambers.

(2) Water samples, to determine test organism levels in the water column, should be taken through the sampling port in the plexiglass microcosm cover. All such samples must be tightly sealed before removal from the microcosm test chamber. Water samples should be taken prior to the water exchange on those days when water exchange is scheduled. (3) Water samples for test organism analysis must be collected: (a) While the turbulence (paddle) system is operating. (b) At a point at least three centimeters from the side of the tank and 10 centimeters below the air-water interface.

(4) Representative samples of the various zooplankton species should be collected once a week (see Section 5.6.6 [4]) to analyze for uptake and accumulation of the test organism . These samples will be collected with the water exchange flask at a rate to preclude zooplankton avoidance. The sample is then passed through the zooplankton collector (Figures 1,7). The water, after passing through the collector, drains into the waste water tank and is decontaminated. The zooplankton sample is rinsed with filtered

sea water and drained into sample bottles. The bottles are tightly capped and decontaminated before removal for analysis. As with all other sampling procedures, access to the zooplankton sampler is gained through the air lock and gloved ports in the waste water chamber.

(5) Potential transport of the test organism to the atmosphere by aerosolization should be tested prior to water exchanges and prior to sacrificing the tanks (5.6.4 [1]) at days 10, 20, and 30. The test organism is collected on a membrane filter inserted in the air vacuum line leaving the plexiglass tank cover (Figure 7). The duration of sampling must be sufficient to collect a representative sample while not exceeding the life expectancy of the test organism on the membrane filter. This will be a conservative estimate of the aerosolization and subsequent gas phase transport because there is no microcosm simulation of wave activity. Methods of analysis to establish the presence of the test organism in aerosols will be dependent upon the characteristics of the test organism.

(6) All samples for test organism analysis should be processed in a time frame consistent with the life expectancy of the test organism in the sample.

5.6.6 Ecological Effects Sampling

(1) During the operation of the definitive test, the plexiglass covers of both the microcosm tank and the test facility (Figure 1) are installed and all biological and chemical samples collected either from the waste water vacuum flask or through the sampling port in the plexiglass cover.

(2) All samples should be taken from at least 10 cm below the

water surface and away from the side of the tank. Sampling must be performed with the paddle system in operation.

(3) The following ecological information is considered to be essential:

(a) Identification of all benthic organisms reported in numbers/m per species.

(b) Zooplankton (including transient larval forms) abundance in numbers/liter per life stage per species.

(c) Phytoplankton abundance in numbers/ml per species.

(d) Ammonia levels in micro moles N/liter.

(e) Ammonia flux rates from the sediment in ugat N/m /hr.

(f) Oxygen uptake rates by the sediment in mg 02/l/m /hr.

Additional analyses such as bacteria counts or growth measures of benthic organism may be performed depending on conditions in the natural system being simulated and the analytical resources available to the investigators.

(4) The type of biological sample, volume of sample, collection frequency, method of collection and analysis are listed below. All samples must be placed in appropriate containers and sealed before being removed from from the microcosm facility.

(a) Two milliliter phytoplankton samples are collected through the sampling port in the tank cover. The algae are counted and identified with methods described by Utermohl (1931).

(b) Zooplankton samples are collected twice weekly. Two liters from the waste water flask are passed through, and the organisms collected on, a 28 um mesh nylon screen (Figures 1,7). The zooplankton is rinsed into a collection bottle and preserved for

numerical counting. Numbers and species composition are determined by examination under a dissection microscope. Wastewater from the 2 liter sample drains into the waste water holding tank for subsequent decontamination and disposal.

(c) Ammonia level in the water column is determined once a week. Twenty milliliter samples, collected with a syringe through the sampling ports, are analyzed colorimetrically (Slawyk and MacIsaac 1972).

(d) Ammonia flux rates from the benthic box cores are measured at the time of tank sacrifice. After the cover is removed water flow to the benthic cylinders is halted for one to three hours (length of the incubation period is inversely related to temperature) The diffuser is removed and a thin plastic cover is floated on the water surface of the benthic cylinder. Water samples of 20 milliliters are collected at the start and conclusion of the incubation period. Analysis for ammonia is identical to (c) above.

(e) Samples for oxygen uptake (or release) are taken at the same time as those for ammonia flux. Fifty milliliter water samples are collected with a syringe and overflowed (to the tank) into 20 ML BOD bottles. A modification of the Azide Modification (Standard Methods, 1976) (0.1 ml of KI and $MgSO_4$ and 0.2 ml of H_2SO_4) is used to prepare the sample. The samples are titrated with a microburet. Results are reported as the flux of oxygen in mg O_2/l/m^2/hr.

5.6.7 Procedure for Final Microcosm Sampling

(1) The plexiglass tank covers are removed from the appropriate tanks, after routine sampling has been performed, on days 10, 20, and 30. Cover removal is accomplished via the gloved ports.

(2) Those microcosm tanks that have been selected for sacrifice and sampling at days 10 and 20 (Section 5.6.4 [1]) and all remaining tanks following the exposure period must have the following samples taken, sealed in bottles, decontaminated and removed from the test chamber.

(a) Triplicate sediment sub-cores (2.5 cm diam. x 10 cm deep) to determine the vertical distribution of the test organism in the benthic substrate. These cores include the sediment and associated biota.

(b) The overlying water from the benthic box is removed with a syringe. The top one-to-two centimeters of sediment remaining in the benthic box are removed and sealed in bottles for subsequent screening.

(c) Estimates of the amount of test organism accumulated on the microcosm surfaces are made at the time of the tank sacrifice. Replicate samples of the suspended fiberglass disks (4.2.1 [7]) are sealed in vials and removed for analysis.

(3) After all samples have been collected and removed from the test chamber, sufficient disinfectant is added to the two tanks and benthic boxes to kill the test organisms therein. These decontaminated tanks are recovered and left untouched until the end of the experimental period.

(4) If a steady state condition of the test organism concentration in the water column is achieved by day 30 the three remaining exposed tanks will be sacrificed and sampled as described in Sect. 5.6.7 to determine the fate and mechanism of effect of the test organism in the affected ecological compartment. Otherwise, the

test, with the remaining six tanks, will be extended to 60 days and sampled as prescribed in Section 5.6.7.

(5) At the conclusion of the experiment, disinfectant is added to the waterbath, all interior surfaces of all test chambers, air locks, replacement and waste water chambers. The covers of the tanks and test chambers can then be removed, water and sediment discarded after growth detection tests show the absence of test organism within the system.

5.7 Waste Disposal

5.7.1 Liquid and solid wastes generated through sampling and water turnover must be collected and held until decontamination treatments have rendered the test organism safe for disposal.

5.7.2 Sufficient volumes of the disinfectant (Section 5.5.5) must be used to decontaminate the water in the exposed microcosm tanks.

5.7.3 Contaminated solid wastes such as sediment, filters, charcoal, ion exchange resin, glassware, gloves, etc, must be disinfected before disposal. If the disinfectant is a toxic material it must be disposed in an appropriate manner.

6. DATA ANALYSIS

6.1 Ecological Effects Analysis

Analysis of variance procedures (Steel and Torrie 1960) and regression techniques (Snedecor and Cochran 1980) should be used to analyze all data. The level of significance is set at the 5% level (a = 0.05) unless otherwise defined. Documentation of all analysis used in the study must be provided.

6.1.1 Test Organism Effects: Perturbed Case

The model of repeated measures, analysis of variance or a completely

random design will be used to detect differences in the 30 day (and if necessary, 60 day) responses due to the test organism relative to the laboratory control set. The specific replicates for the laboratory control set will depend on the results of 6.1.2.

6.2 Test Organism Fate Analysis

The analysis should consist of two phases. First, determine the total biomass (density) of the test organism in the various microcosm compartments (water, surface microlayer, sediment, aerosol and microcosm surfaces). This includes attempts to document the presence of the test organism in the control tanks. A standard regression analysis should be performed on all time series data to determine if the test organism is increasing or decreasing relative to the addition rate. Secondly, the investigators should establish whether the means and associated variances of each compartment are independent of one another. If dependence is found, then appropriate transformations (e.g., Log 10) should be applied so that the independence assumption can be satisfied. Statistical analyses are performed on the transformed data, if necessary, to test for differences between control and exposed sets.

6.3 Fate of Test Organism

Determine the concentration of the test organism in the following microcosm compartments on days 10, 20, and 30.

(a) Surface microlayer

(b) Water column

(c) Sediment core

(d) Selected biota

(e) Microcosm surfaces

6.3.1 Fate Analysis

Test for compartmental differences in the concentration of the test organism from each of the compartments listed in 6.3. Establish a regression line and test for differences over time.

6.4 Transport of Test Organism

Estimate the total biomass of the test organism exported from the gas and liquid compartments via water exchange and aerosolization over the 30 day period. Test for changes in biomass over time.

6.4.1 Transport Analysis

Test for concentration differences of the test organism for each of the compartments listed in 6.3 at days 10, 20, 30, and if necessary 60. Perform the same analysis as above for the integrated concentration of the test organism transported during the term of the experiment. Attempt to develop an expression that will determine the net growth of the test organism over the term of the experiment.

GROWTH = (INPUT - (TRANS + SED + WATER + SML + WALL))

INPUT = The total amount of test organism added during experiment.
TRANS = The total removed during water exchange plus aerosol.
SED = The total found in the sediment.
WATER = The amount remaining in the water column at test conclusion.
SML = The total measured in the surface microlayer.
WALL = The total adhering to tank wall (liquid and gas phases).

6.5 Bioaccumulation

Compute both (a): the concentration of the test organism in either the microcosm animals and/or their specific tissues, in units of numbers/wet weight and (b): the ratio of animal or tissue concentration to medium concentration (Bioaccumulation Factor). Choice of species should be based upon commercial importance

(e.g., hard shell clam or close analogue) or known or suspected linkages to man-based food chains. At least three replicates, (each from separate microcosm systems) of a given species/ concentration combination should be measured if possible. Indicate feeding type in the case of benthic species.

6.5.1 The choice of medium, for bioaccumulation calculations, will be dependent upon the species feeding type. If the medium is sediment, the concentration of the test organism in the top 1 cm of the sediment core should be used to define the exposure level.

6.5.2 Bioaccumulation Analysis

Test for test organism accumulation differences for selected (Section 6.5) species.

6.6 Environmental Assessment of Test Organism

Provide an environmental assessment of the potential fate and effects of the test organism based on the analysis performed above and literature findings.

7. TEST PROTOCOL BIBLIOGRAPHY

American Public Health Association, American Water Works Association, Water Pollution Control Federation. 1975 Standard Methods for the Examination of Water and Waste Water. 14th Edition. New York.

Anonymous. 1984. Biosafety in microbiological and biomedical laboratories. Public Health Service, Center for Disease Control and National Institutes of Health. U.S. Dept. Health and human Services, HHF Publication no.(CCDC)84-8395, 1st ed.

Perez, K.T. 1983. Experimental marine microcosm test protocol and support document: Measurements of the ecological effects,

fate, and transport of chemicals in site-specific marine ecosystems. Accession no. PB83-230854. Technical Information Service, Springfield, VA.

Slawyk, G., MacIssac, I. 1972. Comparison of two automated methods in a region of coastal upwelling. Deep Sea Research, 19:521-524.

Steele, R.D.G., Torrie, B.H. 1960. Principals and Procedures of Statistics. McGraw-Hill Book Co., N.Y. 481 pp.

Snedecor, G.W., Cochran, W.G. 1980. Statistical Methods. 7th Edition. Iowa State University Press, Ames, Iowa.

Utermohl, H. 1931. Neue Wege in der quantitativen Erfassung des Planktons. Verh. int. Verein. theor. angew. Limnol., 5:567-595.

List of Figures

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b) Figure 2 Paddle Drive and Speed Control System

c) Figure 3 Experimental Microcosm

d) Figure 4 Air Control and Supply System for Benthic Pumps

e) Figure 5 Benthic Subsystem

f) Figure 6 Exposure Chamber

g) Figure 7 Microcosm Cover and Zooplankton Sampler

SUPPORT DOCUMENT

Purpose of the Support Document

The purpose of the support document is to provide a general rationale for the specifications and use of the experimental microcosm system to determine the fate, ecological effects, and transport of selected test organisms in coastal marine environments.

Rationale for the Use of Microcosms

The fate and ecological effects of an organism introduced into a given ecosystem are necessarily dependent on both (a) the characteristics of the organism and (b) the physical, chemical, and biological properties and interrelationships specific to that ecosystem. Any evaluation of potential environmental impact, therefore, must be based on data derived with these complexities in mind. Ideally the microcosm of a specific marine ecosystem should be as realistic as possible and allow for the: (1) prediction of ecological effects and test organism fate at the system level of organization, (2) production of verifiable data, (3) standardization and replication among different laboratories, and (4) application of standard statistical techniques for data analysis.

The microcosm methodology described in the Test Protocol is used as an experimental, physical model of a specific portion of a natural ecosystem. Much of the rationale for the design, conditions, and operation of the microcosm system is derived from attempts to duplicate the biotic and abiotic conditions of a natural system (Perez et.al., 1977). This experimental laboratory system meets the criteria listed above and should provide a predictive capability for assessing the potential fate and effects associated with the release of a specific organism into coastal marine environments.

Rationale for Microcosm Design

Much of the rationale for the microcosm's materials and methods are stated in the test protocol or are intuitively obvious. These areas will not be covered in this support document.

It is specified that the exposure and control microcosm systems be physically separated. This is to isolate the control tanks and prevent their contamination with the test organism.

Since the microcosm test protocol is intended to be a site-specific test simulating a marine system, a number of system components and experimental conditions will be dependent on environmental conditions at the site (natural system) being simulated. The physical variables are light (intensity and photoperiod)(Section 5.6.2 [6]), temperature (Section 5.2.6 [7]), water turbulence (Section 5.6.2 [5]), water turnover rate (Section 5.6.2 [10]), and ratio of benthic surface area to water volume (Section 2.1.1 [6]). The biotic components are the endemic water column and benthic communities (Section 5.6.2 [1] and [3]).

In the microcosm, environmental conditions of photoperiod, temperature, turbulence, water exchange rate, benthic surface area, and benthic pumping rate are all established at levels that best duplicate conditions in the natural system. Previous experiments (Perez et al., 1977) have show that the field-derived conditions are essential in order for the biotic responses of the microcosms to be equivalent to those in the natural system. Appropriate light intensity, however, must be empirically determined. Performance of the test at the temperature extremes of the natural system is specified to insure that the test organism is exposed to the full range of expected temperatures. This will allow better estimations of the organism's fate and environmental effects.

The selection of tank volume of 140 liters (Section 4.2.1) is somewhat arbitrary. However, this tank size, and associated box core, does result in a representative benthic component when simulating most coastal and/or estuarine ecosystems. In addition, the inclusion, where applicable, of a commercially important bivalve is insured when the investigator uses this protocol. The primary consideration should be that the tanks be as large as possible while allowing for sufficient numbers of replicates within personnel, space, and/or monetary constraints.

Rationale for Test Procedures

Test water collection and distribution

The protocol requires the use of a water collection method which is nondestructive to planktonic organisms (Section 5.6.2). The application of such a method insures that any divergent behavior of the water column communities between the experimental microcosm and the natural system is due to microcosm effects and not the artifacts of the water collection and distribution methods (e.g., pumping). The simultaneous filling of all microcosm tanks at the start of an experiment (Section 5.6.2. [2]) satisfies a major assumption of the recommended analysis of variance procedure (Section 6.1), specifically, that the experimental units (microcosms) are as identical as possible prior to the application of the treatment conditions (concentrations of the test organism).

Test organism addition

The manner of preparation and addition of the test organism in a stock solution is dependent upon the nature of both the test organism and receiving water. In general, the test organism should be added in a manner similar to that observed or predicted in the natural system.

It is recommended that test organism concentration be established at known or projected field levels.

Sampling

Simultaneous measurements of the test organism and ecological effects are prescribed for a thirty day period (Section 5.6.4 and 5.6.5). This sampling period enables the investigator to establish either the time for the test organism to reach quasi-equilibrium or the degree to which equilibrium is reached within the various physical and biotic compartments of the experimental microcosm. An extension of the experimental time period (Section 5.6.1 [3]) is prescribed when the test organism concentration in the water column has not reached a steady state. This extension allows more time for the test organism to reach equilibrium in the experimental microcosms and, thus, permits more accurate estimates of test organism fate.

Ecological effects

Those measurements required to describe ecological effects (Section 5.6.5 [4]) are dependent on the characteristics of both the contained biotic communities and the test organism. Ideally, a structural and/or functional measure should be made from each trophic level. It is recommended that additional ecological measures be made in those compartments exhibiting significant accumulation of and/or exposure to the test organism.

Ecological fate

Test organism compartments: The test organism is measured within the microcosms to establish whether the test organism is capable of surviving and being transported to adjacent environments as well as bioaccumulating in man based food chains. (Section 5.6.4).

Bioaccumulation of the test organism can result in subsequent ecological

effects and may also be a potential source for human exposure via direct consumption or trophic transfer. It is essential, from a management viewpoint, to be able to quantitatively define the relationship between the amount of test organism added i.e., the input function, to subsequent bioaccumulation levels, trophic transfer, and ecological effects. Analysis of sediment sub-cores is required to determine the vertical profile of the test organism and, thus, exposure concentrations for some of the benthic organisms. Field validation studies have shown that the top one centimeter of the sediment (Section 5.6.1) is where most deposit feeding and associated bioaccumumlation occurs. It is necessary to develop a budget of the test organism throughout the course of the experiment (Section 6.2). This information will indicate survivability of the test organism within the experimental microcosms. The compartments identified as essential to an organism budget are the overlying gas phase, water column, sediment, microcosm surfaces and benthic macrofauna not included in the sediment sub-cores.

Data Analysis

One of the major features of the recommended experimental design and statistical analysis (Section 6.1) is the ability to independently establish the quantitative effect of the test organism for all the ecological variables measured. In addition, the degree of divergent biotic behavior between the control microcosms and the natural system provides a measure of the validity of the microcosm responses to the test organism.

Restrictions of Test Protocol

Scalar dimensions (Section 4.2.1) and simulation complexity (Section 4.1) limit the use and interpretation of results from experimental microcosms. Specifically, the size of the microcosm results in the exclusion of

macrofauna such as fishes and large crustaceans. Since most, if not all, microcosms are so restricted, such a limitation is characteristic of this experimental approach. In addition, the microcosm in its present design does not simulate the effects of ultraviolet light. Because the microcosm light lacks the U.V. component of the natural system, its possible effects on survival, growth, and reproduction of the test organsim, especially if it accumulates at the air-water interface, cannot be estimated.

Support Document Bibliography

Perez, K., Morrison, G., Lackie, N., Oviatt, C., Nixon, S., Buckley, B., and Heltshe, J. 1977. The importance of physical and biotic scaling to the experimental simulation of a coastal marine ecosystem. Helgolander wis Meeresunters. 30:144-162.

Summary

Three laboratory marine systems are presented that may be used to evaluate the impact of genetically engineered and naturally occurring MPCA's on the near-shore environment. The microcosms, one simple with fish, shrimp and two molluscs, one a simulative assemblage of shallow, intertidal organisms including plants and another simulative assemblage representing deeper water communities. The systems differ in complexity; the simple system with four selected species and the simulative systems with populations and numbers of organisms as collected in the environment. They also differ in other factors such as the manner in which water is supplied. The simple system is static, the near coastal system can be static or flowing and the deeper water system is maintained with flowing seawater.

Of the three proposed microcosm tests, only the simple system has been operated with microorganisms. The simulative systems were developed for testing the impact of toxic organic chemicals and the authors believe that these systems must be tested with microorganisms before they are used in routine testing with microbial pest control agents.

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This section contains the following:

1. Methods for conducting multi-species, vertebrate and invertebrate freshwater acute tests with Microbial Pest Control Agents (MPCA's). Gary L. Phipps and Gary W. Holcome, ERL-D, Duluth, MN 55804.

2. A procedure for exposing stream invertebrates to microbial pest control agents in the laboratroy. Terry E. Flum and Richard L. Anderson, ERL-D, Duluth, MN 55804.

3. Methods for a microcosm test to determine survival and ecosystem level effects of Microbial Pest Control Agents. John W. Leffler, Dept. of Biology, Ferrum College, Ferrum, VA 24088; Lyle Shannon, Dept. of Biology, University of Minnesota, Duluth, Duluth, MN 55812; Carolyn L. Thomas, Dept. of Biology, Ferrum College, Ferrum, VA 24088; J. David Yount, ERL-D, Duluth, MN 55804.

4. Methods for establishing microcosms of a aquatic littoral ecosystems for determining safe levels of agent exposure. Paul J. Franco, Oak Ridge National Laboratory, Oak Ridge, TN 37381; Jeffrey M. Giddings, Springborne Bionomics, Wareham, MA 02571.

INTRODUCTION

Interest in microbial pest control agents (MPCA's) for insect control is increasing. Concurrently, there is an increasing need to develop an understanding of the potential for adverse effects from these agents to populations, communities and ecosystems. The need is particularly great in freshwater systems because of the variety of habitats that may receive biological control agents from either direct application or from applications to land adjacent to water.

Microbial control agents are unique when compared to other pest control agents, because thay are alive. Life adds questions of infectivity, pathogenicity and competition to the usual concerns of toxicity, decreases in growth an reproduction and adverse changes in population, communities and ecosystems. In a tiered evaluation system, the first step to develop an understanding of effects is laboratory acute bioassays. The acute tests can be followed by chronic life cycle exposures which examine reproduction, growth and mortality. The final laboratory tests are microcosms, which are designed to simulate outdoor systems. In this report, systems which were developed for chemical toxicity determination are described. They include two laboratory systems that can test many species at one time and two microcosm systems that provide the complexity of interacting populations and allow measurement of system effects.

The first test described in this section is a multi-species exposure system that was designed to provide chemical toxcity data on a variety of species from a single test. In its current design a diluter is used to provide water to 12 exposure tanks. This system may be adapted for specific MPCA use by reducing the number of exposure tanks and altering the diluter or developing other methods to deliver water and maintaining animals in the

system. The key item in this test is the mechanism for exposing eight or more species.

The second test in this section describes an exposure system for stream invertebrates. The exposure tank is a trough, about 10 cm wide and 13 cm deep, that is turned in an oblong form to provide a continuous channel. Physical factors such as flow and temperature are controlled by a circulating pump and a water bath. Direct exposure of animals can be accomplished by enclosing animals in screen containers or by simple release into the tank. The impact of sediments, rocks, or plants on the expression or toxicity of an agent can be tested with this system.

The remaining protocols in this seciton describe methods to produce laboratory microcosms. One system was designed to provide data on ecosystem effects of chemicals but is being applied to questions of survival and ecosystem effects of MPCAs in freshwater systems. The microcosm development is started by collecting biota from natural outdoor sources and establishing animal stocks in the laboratory. These stocks can be long lived, often lasting more than a year which allows tests to be started at anytime. Material from the stock tank is used to prepare the microcosm. The microcosm containers are one liter beakers which contain a sediment, nutrient supplimented water and, after an equilibration period, stable communities of primary producers, consumers and decomposers. The method, shows good reproducibility and has been field validated with chemical toxicants. The small size of the microcosms permits many to be used simultaneoulsy in a small enclosure.

The other protocol in this section describes procedures for establishing freshwater littoral communities in the laboratory with materials collected from natural ponds. This system was also developed to assess the effects

of chemical toxicants on littoral communities. The procedure utilizes 72 liter aquaria as exposure tanks and establishes microcosms by collecting sediment and water from natural, outdoor systems and placing the material with its biota into the aquaria. This system has the advantages of easy assembly, low maintenance and replicability. Because the microcosms are established from natural ecosystems, a site-specific evaluation of effects can be established.

In summary, this section describes four protocols. The first two systems can provide exposure data on many species at one time. The two microcoms systems can provide data on effects and expression of an agent in complex environments. However, a key consideration for all these protocols is that they may be adaptable to MPCA registration uses.

All the systems were developed to test chemicals using the endpoints of death, reproduction or ecosystem changes. None have been extensively tested with MPCAs and all the writers acknowledged that research is necessary before these systems can be used with the same confidence associated with their use in chemical hazard assessment.

The writers have identified seven areas that need to be addressed before high confidence can be associated with MPCA use as follows:

1) Operation of the procedure with a specific MPCA
2) Endpoints
3) Positive Control
4) Quantitation
5) Dosing
6) Containment
7) Decontamination

The primary research goal is to operate these procedures with an MPCA. Because of the variety of MPCAs, it will be necessary to test the procedures with an agent from each group of interest. The results of the first experiments may affect the other research areas which indicate a need for

comprehensive studies. Each procedure has specific endpoints such as death, reproduction changes or system effects. Methods that measure other effects that may be uniquely associated with MPCAs such as infection, pathogenicity, environmental expression, persistence and competition which may lead to species replacement are yet to be developed for use with these freshwater systems. These methods can be developed through research or evaluation of adaptable procedures during the protocol evaluation. The third research need is the addition of a positive control. In the usual bioassay or toxicity test, concentrations can be selected that will cause a lethal effect. Because MPCAs are not general toxicants, many animals may not be sensitive so it is important that the target animal (if aquatic) be tested in the system to show that the system allows an effect to occur. In the multispecies tests, this may be done by adding a container with target animals to the system. In the microcosm tests, the target animal could be added to a test unit or a separate positive control test unit could be established to verify that the agent, at the test concentration, was causing the observed effect. Areas four through seven point to specific questions that must be answered before the procedures can be widely used. All risk assessments require information of the exposure concentration and effect relationship so quantitation methods for each system are necessary. The last areas, dosing, containment and decontamination are included to show their importance and the need to have methods available before the testing of an MPCA.

METHODS FOR CONDUCTING MULTI-SPECIES, VERTEBRATE AND INVERTEBRATE FRESHWATER ACUTE TESTS WITH MICROBIAL PEST CONTROL AGENTS (MPCAS)

by

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1. SCOPE.

This multi-species test procedure was designed to simultaneously test 8-12 freshwater vertebrates and invertebrates against toxicants. The toxicants include industrial organics, pesticides and metals and the procedure has been used successfully to produce LC50 data on selected species (Phipps et al., 1985). Since it has not been used for testing Microbial Pest Control Agents (MPCAs), several modifications will be suggested to adapt the procedure to those unique MPCA testing needs. This procedure presumes a working knowledge of aquatic toxicity testing so many basic concepts will not be included. This procedure, used a modified Mount-Brungs diluter (1967) with 5 experimental toxicant concentrations and a control, all duplicated. MPCA testing, however, is different in that the need is to determine whether a concentration-independent effect is caused by the MPCA, therefore any apparatus which will provide an exposure concentration and a control is satisfactory.

2. APPLICABLE DOCUMENTS.

2.1 1976 Annual Book of ASTM Standards, Part 31:

D8888 Oxygen, dissolved in water

D1067 Acidity and alkalinity

D1125 Conductivity

D1126 Hardness

D1293 pH

D1426 Ammonia

E729-80 Conducting acute tests with fishes, macroinvertebrates and amphibians.

2.2 EPA-660/ Ecological Research Series

3-75-011 Acquisition and culture of research fish: Rainbow trout, fathead minnows, channel catfish, and bluegills.

3-75-009 Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians.

3. SUMMARY.

This procedure differs from standard acute toxicity tests in a few key areas as follows:

3.1 The MPCA target organism may be tested simultaneously with a diverse variety of nontarget organisms that might be found in the same body of water or area.

3.2 Several vertebrate and invertebrate species are tested simultaneously, yielding considerably more data for the time, effort, and money expended.

3.3 All species are exposed in the same test chamber, in the same water column, assuring uniform exposure conditions for all tested species thereby decreasing test-to-test variability.

3.4 Warm and cold water species are tested together at 17° C, which is a recognized standard warm water test temperature (ASTM E729, 1980), and within the physiological optimum for rainbow trout (Lichtenheld 1966; Lavrosky 1968; Dickson and Kramer 1971; McCauley and Pond 1971; Hokanson et al. 1977).

3.5 A self-starting siphon was used (Benoit and Puglisi 1973) which varied the height of the test water in the exposure chamber (Fig. 1) which effects water exchange in the Daphnia and Chironomid exposure beakers (Fig. 1) making the MPCA concentration in the beakers the

same as that in the exposure chamber.

3.6 It was easier to distinguish changes in behavior and to determine the most sensitive species.

4. SIGNIFICANCE.

4.1 This test was designed to produce an acute aquatic toxicity data base for a minimum of time, effort and finances. In addition, the test organisms were selected for their diversity in representing the freshwater community, their ease of procurement, availability and ease of culturing.

4.2 Because all of the organisms in each concentration were tested in the same water columnm, species differences in toxicty became very apparent, the more sensitive species were easily identified, and behavioral differences could be observed.

5. QUALITY CONTROL.

5.1 Organisms.

It is recommended that organisms be brought into the laboratory and acclimated to the dilution water for at least 2 weeks. During this time mortalities should be less than 10 percent in 2 weeks. If mortalities exceed this limit, the organisms should be treated for disease, destroyed, or new ones procured. After treatment, the organisms must be reacclimated.

5.2 Control Mortality.

Control mortality should never exceed 10 percent per species per test. A test must be repeated if mortality is greater than 10 percent. Practically speaking, however, if any control mortality takes place, serious scrutiny of the test should take place to isolate the cause because the quality of the test is jeopardized.

5.3 Test Conditions.

Test conditions must be maintained as close to constant as possible. Strict attention must be given to temperature, dissolved oxygen, fighting and any other variable which affects the well-being of the organism. The ideal test has only one variable between the control and experimental treatments, so all the other variables must be controlled as closely as possible.

6. FACILITIES.

The test and animal holding systems must be located in a laboratory with adequate physical characteristics which include:

6.1 Water Supply.

A water supply of satisfactory quality for aquatic testing must be available for the holding and test systems. The water must be tested for adequacy using methods described by APHA (1980).

6.2. Electrical Supply.

An electrical supply for powering testing equipment, dosing pumps and fluorescent lights must be protected by ground-fault-interrupt to protect the researchers from electrocution.

6.3 Air Supply.

An-oil free air supply for aeration of the fish tanks and brine shrimp cultures must be supplied.

6.4 Temperature Control.

Adequate control (± 2° C) of room temperature is essential to maintain uniform test temperatures (± 1° C) in static test systems. Water temperature control of dilution water should be sufficient to maintain the test water temperature within ± 1° C.

6.6 Ventilation.

Negative pressure ventilation must be provided to assure the protection of the researchers from MPCAs or their products.

6.6 Effluent Water Treatment.

Facilities should include a water treatment system to sterilize the effluent to avoid an accidental environmental release.

6.7 Sterilizer.

A sterilizer of adequate size to accommodate exposure tanks and dosing equipment is essential.

7. EQUIPMENT.

7.1 Dilution Apparatus.

Since MPCA testing uses only one toxicant concentration, a standard dilution apparatus is not needed. A variety of apparatuses listed in EPA-660/3-75-009 section No. 3 (1975) should work for MPCA testing. The Mount-Brungs diluter (1967) may be easily modified to give the one experimental concentration and control, by disconnecting 4 of the 5 experimental concentrations.

7.2 Exposure Tanks (Fig. 1).

Glass aquaria measured 60 x 30 cm deep with a 17 cm standpipe and had a volume of 30.6 L. Aquaria were divided into six 16.6 cm x 15.0 cm compartments and one 10.0 x 30.0 cm compartment with stainless steel dividers. The standpipe located in the 10 x 30 cm compartment had a self-starting siphon which varied the water level in the tank by 2.5 cm to allow water exchange in the *Daphnia* and Chironomid chambers.

7.3 Daphnia and Chrionomid Exposure Beakers (Fig. 1).

Beakers were 250 ml in volume. Two holes, 19 mm in diameter, were

drilled opposite each other with their centers 2 cm up from the bottom. These holes were covered by stainless steel screen which was silicone-glued to the outside of the beaker. The screen-covered holes effected water exchange in the beakers without the escape of organisms. A wire was attached (Fig. 1) to the beaker and the beakers were hung in the exposure aquaria so that the lowest water level in the aquarium was 3 cm above the bottom of the beaker.

7.4 Snail Exposure Chambers.

The exposure chambers consisted of stainless steel screen cylinders 10 x 3 cm diameter, closed on one end by stainless steel screen and on the other by a No. 7 neoprene stopper. The chambers prevent the snails from leaving the water during the testing.

8. PROCEDURES.

8.1 Test Preliminaries.

Once the experimental concentration of MPCA has been determined, the MPCA stock concentration must be determined and the stock prepared. The diluter is then started and the experimental tanks filled with MPCA-laden water. The test begins when the species that are tested for 96 hours are counted out and randomly distributed to the exposure chambers.

8.2 Test Start, Day 1

8.2.1 The test started at hour zero on day one when the species were introduced into the different screen chambers in the exposure chambers. Records must be kept of the following: (1) Mortalities for each species at least once every 24 hours. (2) Agent information such as purity, source, lot number, stock concentration and nominal or calculated exposure concentrations. (3) Water temperature.

(4) Dissolved oxygen. (5) Test start date and time. (6) Any other pertinent information.

8.2.2 After the test is started with the species that will be tested for 96 hours, the Daphnia that will be tested for 48 hours must be cultured. Adult Daphnia magna are isolated and fed, and the time of isolation is noted. Exactly 24 hours later the adults are removed. The resulting young Daphnia are then counted into the exposure beakers and hung in the exposure tanks.

8.2.3 The third to fouth instar Chironomid larvae are placed into exposure beakers which had about 1 ml acetone-washed quartz sand previously spread on the bottom. In addition, about 1 ml of midge food is placed into the beakers. Once the larvae are in the beakers, they should be set aside for 24 hours to acclimate and to build tubes.

8.3 Day 2 Testing.

On day 2 or 24-30 hours after the test is started, the Daphnia and midges, as well as any other organism that are tested for 48 hours, are placed into the exposure chambers. A mortality count is made on the other species and the dead are removed. The water temperature is recorded. Snails are counted at 96 hours. Daphnia and Chrionomids are counted only at 48 hours.

8.4 Day 3 Testing.

On day 2 or 24-30 hours after the test is started, the Daphnia and midges, as well as any other organism that are tested for 48 hours, are placed into the exposure chambers. A mortality count is made on the other species and the dead are removed. The water temperature is recorded. Snails are counted at 96 hours. Daphnia and Chrionomids are counted only at 48 hours.

8.4 Day 3 Testing.

Mortalities are counted and the dead are removed. Water chemistry measurements such as dissolved oxygen, alkalinity, pH and hardness are determined and recorded. The water temperature is recorded. Exactly 48 hours after the organisms were introduced into the test, *Daphnia* immobilization and Chironomid and *Daphnia* mortalities are determined for each test beaker and recorded.

8.5 Day 4 Test Termination.

At exactly 96 hours after the start of the test, the final count is made on all species tested for 96 hours. The snails are removed from their exposure chambers and mortalities are counted and recorded. To determine mortality, snails should be inspected with a binocular dissecting scope and the foot probed with a blunt instrument about paper clip wire size to stimulate movement. After mortalities are counted, 10 control fish of each species should be ice-anesthetized and weighed to determine fish size for each species. Removal and disposal of the remaining fish in the test now takes place, and the test system is cleaned in preparation for the next test.

REFERENCES

American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1980. Standard methods for the examination of water and wastewater, 15 edn. Washington, DC.

American Society for Testing and Materials. 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians, E279-80. Philadelphia, PA.

Benoit, D.A. and F.A. Puglisi. 1973. A simplified flow-splitting chamber and siphon for proportional diluters. Water Res. 7:1915-1916.

Biesinger, K.E. and G.M. Christensen. 1972. Effects of various metals on survival, growth and reproduction of Daphnia magna. J. Fish. Res. Board Can. 29(12):1691-1700.

Brauhn, J.L. and R.A. Schoettger. 1975. Acquisition and culture of research fish: Rainbow trout, fathead minnows, channel catfish, and bluegills. Ecological Research Series No. EPA-660/3-75-011. U.S. Environmental Protection Agency, Corvallis, OR. 54 pp.

Dickson, I.W. and R.H. Kramer. 1971. Factors influencing scope for activity and active and standard metabolism of rainbow trout (Salmo gairdneri). J. Fish. Res. Bd. Can. 28:587-596.

Hokanson, K.E.F., C.F. Kleiner and T.W. Thorslund. 1977. Effects of constant temperatures and diel temperature fluctuations on specific growth and mortality rates and yield of juvenile rainbow trout, Salmo gairdneri. J. Fish. Res. Bd. Can. 34:639-648.

Lavrosky, V.V. 1968. Raising of rainbow trout (Salmo gairdneri) together with carp (Cyprinus carpio) and other fishes. In: Proc. FAO World Symp. on Warm-Water Pond Fish Culture, ed. by T.V.R. Pillay, FAO (FAOUN) Fish Rep. 44(5):213-217.

Litchtenheld, R.W. 1966. Effect of light, temperature, and gamma radiation on the locomotor activity of juvenile steelhead trout, Salmo gairderni. Ph.D. thesis, University of Washington.

McCauley, R.W. and W.L. Pond. 1971. Temperature selection of rainbow trout (Salmo gairdneri) fingerlings in vertical and horizontal gradients. J. Fish. Res. Bd. Can. 28:1801-1804.

Mount, D.I. and W.A. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Res. 1:21-29.

Phipps, G.L. and G.W. Holcombe. 1985. A method for aquatic multiple species toxicant testing: Acute toxicity of 10 chemicals to 5 vertebrates and 2 invertebrates. Environ. Pollut. (Series A) 38:141-157.

U.S. Environmental Protection Agency: Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians, Ecol. Res. Ser., EPA-660/375-009, NTIS No. PB242105 (National Technical Information Service, Springfield, VA 22161).

TABLE 1

SPECIES TESTED

Species	Source	Lifestage	Size or Age	Holding Tank Volume	Holding Temperature	Food Type	Feeding Frequency	Number Tested Chamber
Fathead minnow *Pimpephales promelas*	Lab Culture	Juv.	6-8 wks. 0.3-0.8g	57L	20°C	*Artemia nauplii*	2/daily	10
Channel catfish *Ictaluarus punctatus*	Commercial Hatchery	Juv.	1-40g	530L	17°C	Dry food[2]	2/daily	10
Bluegill *Lepomis macrochirus*	Commercial Hatchery	Juv.	0.2-2g	530L	17°C	Frozen Brine Shrimp	2/daily	10
Goldfish *Carassius auratus*	Lab culture or Commerical Hatchery	Juv.	1-20g	57L	17°C	Frozen Brine Shrimp	2/daily	5-10
White sucker *Catostomus commersoni*	Commercial Hatchery	Juv.	Approx. 1-15g	530L	17°C	Dry Food	2/daily	5-10
Chinook salmon *Oncorhynchus tshawytscha*	Commercial Hatchery	Juv.	3-7g	530L	15°C	Dry Food	2/daily	5-10
Rainbow trout *Salmo gairdneri*	Commercial Hatchery	Juv.	0.5-20g	530L	15°C	Dry Food	2/daily	5-1
Crayfish[1] *Onconectes immunis*	Commercial Hatchery	Juv.	2-4g	530L	17°C	Dry Food	2/daily	10
Leech *Nephelopsis obscura*	Bait Dealers	Juv.	≤ 1 year	57L	17°C	Fish & Liver	1/daily	10
Snail *Aplexa hypnorum*	Lab Culture	Adult	1-2 month	57L	20°C	*Artemia nauplii*	2/daily	10
Daphnia magna[3]	Lab Culture	1-2 instar	0-24	2L	17°C	*Daphnia*[4] food	1/daily	10
Midge[3] *Canytarsus dissimilis*	Lab Culture	3-4 instar	7-8 day	2L	17°C	Daphnia food	1/daily	10
African Frog *Xenopus laevis*	Lab Culture	Tadpole	1-4 weeks	57L	20°C	*Artemia nauplii*	1/daily	10

1 May be held in same tank as bluegills.
2 Made to U.S. Fish and Wildlife Specifications.
3 Reared in static conditions.
4 Biesinger and Christensen 1972.

A PROCEDURE FOR EXPOSING STREAM INVERTEBRATES TO MICROBIAL PEST CONTROL AGENTS IN THE LABORATORY

by

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1. SCOPE.

1.1 This method, developed from experience obtained in toxicity testing of stream invertebrates, has been used to examine the impact of biotic and abiotic factors on the distribution of a Microbial Pest Control Agent (MPCA). The primary unit in the system is a stainless steel trough and a circulating pump. Each trough is about 2 meters long, 13 cm deep and 1½ cm wide. The ends of the trough are connected and the system is formed into an oval with an overall length of about 92 cm. The trough is filled to a depth of 10 cm and the pump circulates the water. The stream may be used as a simple bioassay chamber by enclosing the test animals in screen containers suspended in the flow. The stream may also be used for more complex simulations. The complex studies would be accomplished by the addition of sediment, rocks or other debris and animals would not be enclosed but allowed to move freely in the system.

1.2 Successful operation of this procedure assumes a knowledge of toxicity testing and an understanding of the techniques and procedures described in the following texts:

1. Standard Methods for the Examination of Water and Wastewater, 1985.
2. 1976 Annual Book of ASTM Standards, Part 31:

D888	Oxygen, dissolved in water
D1067	Acidity and Alkalinity
D1125	Conductivity
D1126	Hardness
D1293	pH
D1426	Ammonia
E729-80	Conducting acute tests with fishes, macroinvertebrates and amphibians

3. U.S. EPA Ecological Research Series.

660/3-75-009 Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians,

2. SUMMARY.

Wild-caught or cultured invertebrates are enclosed in screen containers and exposed in a laboratory stream system. Flow rates and temperature are controlled and animals can be maintained for at least 30 days, if food is provided. Effects criteria may be death or animals can be sampled for analysis by microscopy or other techniques.

3. SIGNIFICANCE.

3.1 Lotic environments range from springs to shallow streams to rivers. These environments contain a range of habitats that can receive inputs of MPCAs from planned and accidental events. Unfortunately, stream invertebrates are not often used as test animals in the laboratory because of their need for flowing water. This procedure describes a contained, controlled-flow test system. It can be used as a multiple species bioassay chamber or as microcosm for more complex environmental testing. It is small, low cost and all equipment can be reused.

4. APPARATUS.

4.1 Facilities. A holding and a test area are needed in which the animals used in the test can be cultured in the laboratory, collected from natural habitats or obtained from commercial sources. If animals are cultured, then the systems used in the culturing will be adequate or can be adapted to maintain the collected or purchased animals. If the testing laboratory does not culture invertebrates, then holding facilities must be established. Designs and conditions

for rearing and holding are available in reports in section 1. Both facilities should provide an adequate water source, temperature control, and electricity for lights, timers and pumps. In addition, the test area should provide a clean section for test set-up and an adequate waste treatmeant and clean-up facility.

4.2 Test System Apparatus.

4.2.1 Stream Chamber. -The streams are contained in stainless steel troughs, each about 2 meters long, 13 cm deep and 11 cm wide. The ends of the trough are connected and the system is formed into an oval with an overall length of 92 cm. In this form, each stream has a 60 cm straight section and curves on each end that have a radius of 16 cm.

4.2.2 Temperature Control. Stream invertebrates may be exposed at temperatures that are less than 20° C so systems that can maintain lower than room temperatures must be developed. Options include cooling the test area air or using a refrigerated water bath.

4.2.3 Light. Light is provided by wide spectrum fluorescent lamps at an intensity of 270 lux (25 foot candles). A combination of Gro-Lux® and Vita-Lite® bulbs has been used successfully.

4.2.4 Circulation. Water circulation in the trough is provided by a submersible pump. In our system, a water bath was used and the pump was located in the water bath. This was necessary because the pump can heat the exposure water. The pump inlet line was constructed from PVC pipe and positioned just below the surface on the side opposite the outlet pipe. Water that passed through the pump passes through an adjustable float gauge that allows control of flow rates. The water returns to the trough through a dispersing

orifice located at the water surface, parallel to the trough bottom. In our system, the oulet was constructed from a PVC plumbing "T" that was plugged on the ends of the "T" and had 3, 2 mm holes drilled to allow the water to be forced out under pressure.

4.2.5 Screen Containers. Stainless steel screen containers are recommended because of their durability. The containers can be constructed in either cylinder or cube form with largest mesh size that retains the test animals. The design should include either a built-in cap or have another provision for capping. The containers are placed on the bottom of the trough or suspended by hooks from the side of the trough. The containers should extend above the water surface at lest 3 cm.

5. WATER.

Test waters commonly used in bioassays or toxicity testing are from wells or the surface. The water should be uncontaminated and of constant quality. Specifications for the test water are provided in the references in Section 1. A common guide for water quality is its suitability for rearing *Daphnia*. If *Daphnia* can be reared or if the water is adequate for culturing other invertebrates, it is assumed be adequate for use in this procedure. Water should be added to replace evaporative loss. The water can be slowly added by pouring through a tube directly in front of the outlet.

6. TEST ORGANISMS.

Selection processes for lotic test organisms should include a prediction of what populations will be exposed if the agent is directly applied for controlling an aquatic pest or what aquatic populations will be exposed if the material is applied to a

terrestrial system. If the preferred nontarget animals are not available then substitutions should be closely related to the selected animals. There are few commercial sources of stream invertebrates and local collections may be the best source of animals.

6.1 Collection and Laboratory Acclimation. Collection techniques for invertebrates include kick-nets, seines, screens and dredges (Merritt, Cummins and Resh, 1978). After collection, the animals should be quickly transferred to the laboratory. Special care should be given to maintaining the dissolved oxygen near saturation and providing a current through a portable aerator. A record of the collection and maintenance procedures is essential. Animals should be kept at the test temperature for at least 1 week before testing. Dead, diseased or damaged individuals should not be used.

6.2 Experimental Design. A minimum test would include one exposure concentration and a control that would receive no additions of the MPCA. A position control would be accomplished by a container in the exposure stream that contains the target organism (if aquatic). This is included to assure that the agent is toxic at the test concentration. Each test is done in duplicate so 4 troughs would be required. If additional concentrations were needed, two troughs would be added for each concentration.

7. PROCEDURE.

7.1 Preparation. Some of the preparation for an exposure must be completed one or two weeks before the test so the procedure will be presented chronologically:

-14 days - Test animal collections should be completed at least one week before the test to provide time for acclimation to temperature, food and usually a different water.

-7 days - All collected animals should be acclimated to the test conditions. Begin daily checks for animal health. Monitor dissolved oxygen, temperature and pH of the holding system. If reared animals are being used, then monitor those systems to assure similarity to the collected animals holding condition.

-3 days - The troughs should all be filled and operating by this day. Dissolved oxygen, pH and temperature should be checked each day. These values must be stable for at least 2 days before the animals are added. All the screen containers should be cleaned and sterilized.

-2 days - Distribution of the test animals. Two containers of 10 animals each are necessary for each trough so a total of 4 containers (40 animals) are needed for the minimum test. To prepare the containers for 1 species, carefully remove the animals from the holding/rearing tank and transfer to a shallow white enamel pan with enough water to allow easy movement of the animals. Transfer small animals with a pipet or large animals with a piece of screen shaped into a scoop to each of 4 beakers. Continue the process until each beaker has 10 animals. Place labeled screen containers into each trough and randomly select a beaker and gently pour the animals into the screen container. Continue until all animals in each beaker are transferred and then count the animals in each screen container. Repeat this process for each species until all the test animals are in the test troughs. Most species

will not leave the water but caps may be required on the screen enclosures.

-1 day - Check all the screen enclosures for number and condition. Replace any that do not appear healthy. Select the streams that will receive an MPCA.

0 day - Check all the animals before adding the MPCA. The methods for adding the test agent depend on the formulation (liquid, powder, or granule) and other special considerations that cannot be forseen. Before the addition, the dissolved oxygen and pH should be measured. After addition, these same measurements should be made at intervals that will describe their changes during the test. Also, a schedule for monitoring the agent concentration should be prepared before the exposure begins and should be followed throughout the exposure.

+1 day - On each day, the animals in the screen enclosures should be counted and recorded along with any observations on fitness. If a sampling schedule or a decision that any "impaired" individual would be removed for analysis has been made, those animals can be removed at this time.

The animals should be checked daily until the test is complete. Exposures up to 30 days have been successful in this system.

7.2 Temperature. The temperatures should be maintained within $\pm$ 1° C. Temperature measurements should be continually recorded. Minimum requirement is a daily measurement.

7.3 Dissolved Oxygen. Aeration should not be necessary because of the recirculating system. Dissolved oxygen should be measured before the test and daily during the exposure. At least one set

of measurements should be made throughout a 24 hour period to monitor for sags in oxygen content. A preferred system would use a probe that measures oxygen content and an automatic recording system.

7.4 pH. The pH should be monitored on the same schedule as the dissolved oxygen. A continually recording system is preferred to spot sampling.

7.5 Feeding. Feeding requirements may be specific for some animals and those foods should be provided. For many stream invertebrates, soaked hardwood leaves (maple, aspen, alder, birch) are acceptable. The leaves are most acceptable if soaked for up to 30 days in control water. The leaves can be added directly to the screen enclosures and replaced when only the skeleton remains. If the containers are suspended, the fecal material and debris will fall to the bottom of the trough.

7.6 Concentration Monitoring. A critical part of the test is the concentration of the test agent in the system. Before the test begins, a prediction of the probable distribution of the agent in the system must be completed and a sampling schedule must be devised to monitor these locations. After the test starts, the schedule may change.

8. QUALITY ASSURANCE.

8.1 Criteria for accepting a test rests on definite statements and good judgement. Chemical testing criteria include:

8.1.1 At least 80% of the control animals survive.

8.1.2 Temperature deviation does not exceed 1° C.

8.1.3 Dissolved oxygen does not drop below 40% saturation.

8.1.4 pH does not deviate more than 1 pH unit.

If a test is awry, good judgment requires that it be terminated and repeated. It is critical, even in those tests, that the observations be used to improve the next test.

9. OPTIONS.

The recirculating trough can also be the basis for a stream microcosm. The basic system has been adapted to examine the fate of an MPCA in a more complex system and the impact of nontarget animals on the fate and distribution of an MPCA. The basic system was altered by the addition of a sand substrate and rocks were simulated by unglazed clay tiles. In the studies, beach sand was sieved through a 250 um screen and rinsed in water before it was added to the troughs. The sand was added to a depth of about 3 cm. The unglazed tiles were used to simulate natural rock. Each commercial tile was quartered to produce small squares, about 7.5 cm on a side. The tiles were inserted into the sand at about a 45 degree angle, leaning away from the flow. About one-half of each tile remained above the sand. The tiles were centered to allow flow on both sides. Twenty-two tiles were placed in each trough, about 10 cm apart. In the sand-tile system, free stoneflies (*Pteronarcys*) have been successfully maintained for 30 days. Other nonpredaceous invertebrates could also be added to this system.

10. ADDITIONAL INFORMATION.

Many references are in the reports mentioned in Section 1. Additional information about testing and maintenance of stream invertebrates can be obtained from the following sources:

Nebeker, A.V. and A.E. Lemke. 1968. Preliminary studies on the tolerance of aquatic insects to heated waters. J. Kansas Entomological Society 41(3):413-418.

Nebeker, A.V. 1972. Effect of low oxygen concentration on survival and emergence of aquatic insects. Trans. Am. Fish. Soc. 101(4):675-679.

Anderson, R.L. 1982. Toxicity of fenvalerate and permethrin to several nontarget aquatic invertebrates. Environ. Entomol. 11(6):1251-1257.

Anderson, R.L. and P. Shubat. 1984. Toxicity of flucythrinate to Gammarus lacustris (Amphipoda), Pteronarcys dorsata (Plecoptera) and Brachycentrus americanus (Trichoptera): Importance of exposure duration. Environ. Pollut. (Series A) 35:353-365.

References

Merritt, R.W., Cummins, K.W., and V.H. Resh. 1978. Collecting, Sampling and Rearing Methods for Aquatic Insects. In: An Introduction to the Aquatic Insects of North America, Ed. R.W. Merritt and K.W. Cummins, Kendall/Hunt Publishing Co., Dubuque, Iowa. pp. 13-31.

METHODS FOR A MICROCOSM TEST TO DETERMINE SURVIVAL AND ECOSYSTEM LEVEL EFFECTS OF MICROBIAL PEST CONTROL AGENTS

by

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1. SCOPE

1.1 Description of test. This protocol is designed to provide data on survival and ecosystem-level effects of microbial pest control agents (MPCAs) introduced into a freshwater environment. Such microbial agents are those subject to test regulations under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). The EPA will use these data to assess the ecological hazards an introduced microorganism presents to the freshwater environment.

The test systems are generic aquatic microcoms containing stable communities of primary producers, consumers and decomposers. Changes are monitored at the ecosystem-level to provide a measure of effect that is integrated across population and community boundaries.

1.2. Justification for test. The EPA has recognized for some time that evaluation of chemical hazard to environmental systems requires testing at levels of the ecological hierarchy above the single species level (Environmental Protection Agency, 1979). A recent study by the National Academy of Science (1981) concluded that tests at the population and ecosystem levels as well as single species tests "are needed before sound judgements can be made about the potential environmental hazard of any chemical." The same conclusions apply to evaluations of the survival and effects of MPCAs.

This protocol provides data which are complementary to those obtained from single species tests. The main advantage of this protocol is that it represents an increase in "ecological realism" over single species tests (Schneider, 1981). It permits evaluation of effects on functional properties at the ecosystem level of

organization. Effects due to species interactions, trophic organization, and biogeochemical cycling can be asesessed. Sensitivity of microcosms to microbial agents may vary greatly, being either greater or less than sensitivity values obtained from single species tests.

The microcosms of this protocol do not simulate any natural system, but do posses certain functional characteristics common to all freshwater lentic systems. Since many kinds of organisms are absent from the microcosms, commercially important species of fish and shellfish should be tested independently. The test outlined in this protocol should be used as a first approximation in identifying microbial agents that are persistent and/or potentially harmful to environmental integrity.

2. SUMMARY OF TEST

The purpose of the microcosm test is to determine fate and ecosystemlevel effects of agents introduced into aquatic environments. This test assumes that certain ecosystem-level functions are common to all ecosystems and are not the result of specific taxonomic groups present in a particular system.

2.1 Test systems. The test systems are aquatic micro-ecosystems established in one liter beakers and maintained in an environmental chamber.

2.2 Durations of test. A complete test requires twelve weeks. Microcosms are constructed and allowed to develop and mature for six weeks. The microbial agent is then added and monitored for an additional six week period.

2.3 Measurement of effects. Mature microcosms are inoculated with

varying densities of the agent to be tested. Effects of the organism are determined through periodic measurements of pH, Eh (redox potential), dissolved oxygen (DO) and ^{14}C glucose decompositon. These variables are all correlates to such ecosystem processes as primary production, community respiration, secondary production, decompositon and nutrient flux.

2.4 Determination of survival rates. Survival rates of the organism are determined through periodic enumeration of the agent.

2.5 Output. This test can provide estimates of minimum effect densities and minimum survival densitites for the microbial agent tested. It also provides data on survival rates and the magnitude of environmental effect.

3. SIGNIFICANCE.

The data generated from this test can be used to develop a first stage assessment of the ecological hazard an organism would present to aquatic environments. The results of this test together with other early assessment data can determine the need for further environmental testing.

3.1 Importance of ecosystem level testing. Ecosystem level tests provide an intermediate between single species effects tests and full scale environmental tests of survival and effects. As Schneider (1981) and others have noted, they yield a more "ecologically realistic" estimate of fate and effects than do single species tests.

3.2 Significance of variables measured. This protocol yields minimum effect densities for the test microorganism expressed in terms of the following ecosystem level parameters: pH, Eh, net daytime community

production (oxygen gain), night community respiration (oxygen loss), and decomposition rate of orgnaic matter. The procedure also determined the minimum densities necessary for survival of an introduced microbe.

A deviation in pH and Eh may have significant impact on an aquatic ecosystem. Changes in the equilibrium of chemical species may alter normal nutrient dynamics with resultant impacts on the biota (Schindler, et al., 1980).

A deviation in the autrophic and heterotrophic components of an ecosystem could alter food chains with resultant impact on species important to human commerce and recreation. Because of the laws of thermodynamics, energy is lost at each step of a food chain. A decrease in autotrophy at the base of a grazing food chain may ultimately lead to a reduction in game fish biomass several levels removed. Similarly an increase in heterotrophy, most notably by bacteria, may also decrease the food supply passing to game fish at the top of detrital food chains. The decrease in dissolved oxygen resulting from increased heterotrophy may also adversely affect game fish such as trout.

A deviation decomposition rate could adversely affect the nutrient cycles of an aquatic system by altering the mineralization rates of organic matter. An excess of nutrients could lead to algal blooms and lowered dissolved oxygen concentrations which stress game fish of commercial or recreational importance. A deficit in available nutrients could reduce primary productivity. This effect could be passed through the food chain resulting in reduced biomass of commercial or recreationally important organisms.

4. DEFINITION OF TERMS.

4.1 Microcosm: A small laboratory aquatic ecosystem containing primary producers, grazers, detritivores, and decomposers and displaying the biogeochemical cycling found in natural ecosystems.

4.2 Ecosystem: A region of the physical environment and its associated organisms.

4.3 Community: A unit composed of all the populations of different species occupying a given ecosystem.

4.4 Population: A unit composed of all the individuals of a single species within a given ecosystem.

4.5 Primary Producer: An appropriate plant species. An organism that obtains its energy from sunlight through photosynthesis (i.e., an autotroph). In these microcosms all the primary producers are algae.

4.6 Grazer: An organism which obtains its energy by feeding on primary producers.

4.7 Heterotroph: An organism which obtains its energy from organic compounds manufactured by primary producers.

4.8 Detritivore: An organism which feeds on dead oraganic material and its associated microbial community.

4.9 Decomposer: An organism, usually a bacterium or fungus, which obtains energy by breaking down the remains or waste products of other organisms.

4.10 Benthic: A organism whose activities are primarily confined to the substrate.

4.11 Planktonic: A small, nonmotile or weakly motile organism which spends most of its time suspended in the water column.
of microbial test agent.

4.12 Treatment: A group of microcosms treated with an identical density

4.13 Oxygen Gain: The daily increase in dissolved oxygen caused by algal photosynthesis.

4.14 Oxygen Loss: The overnight decrease in dissolved oxygen in response to plant and animal respiration.

4.15 Heterotrophic Activity: An indicator of the density of the microbial population determined by the rate at which ^{14}C glucose is decomposed.

4.16 Minimum Effect Density: The lowest treatment level at which a microbial agent causes a measureable ecosystem effect.

4.17 Minimum Survival Density: The lowest treatment level at which an introduced microbial agent can survive in a microcosm system.

4.18 Maximum Hazard Test: A test to determine the effects of an MPCA on a nontarget species when appled at several times the manufacturers' recommended application rate.

5. APPARATUS.

5.1 Facilities

5.1.1 Growth Chambers. Microcosms are maintained in an environmental chamber under controlled conditions in order to enhance replicability and reproducibility.

5.1.2 Temperature Requirements. Temperature is maintained at 20° C within a $\pm$ 1° C range.

5.1.3 Light Requirements. The light cycle is set so that there are 12 hr of darkness and 12 hr of light daily.

Lighting is obtained from "cool light" fluorescent lights. The lamps are arranged throughout the chamber to provide even lighting at an intensity of about 500 f.c. (5381 lumens/M^2).

Microcosms are distributed among all shelves of the chamber and

are spaced to receive equal lighting.

One replicate microcosm from each treatment is placed on each shelf of the chamber. This creates a randomized block design, reducing variability caused by environmental gradients within the chamber. All microcosms assigned to a particular shelf are randomly situated on that shelf.

All microcosms on a particular shelf are moved to another shelf within the chamber twice weekly. This rotation of microcosm within the chamber at regular intervals also reduces within-chamber variability.

Microcosms remain in the environmental chamber at all times except during brief sampling periods.

5.2 Microecosystems

5.2.1 Vessels. The microcosm container is a one liter pyrex beaker covered with a large glass petri dish cover. Because of the lip on the beaker, the system is not completely sealed and gas exchange with the atmosphere can occur. The cover prevents contamination and minimizes the chance for acccidental release of the agent being tested.

Prior to use, the microcosm beakers should be washed according to the procedure in 10.1.2.

5.3 Monitoring Instruments

5.3.1 pH and Eh. A pH selective ion meter with a multiple electrode switch is used to measure pH and Eh. The meter should permit pH readings to 0.01 pH unit and Eh readings to 0.1 millivolt. Glass combination pH electrodes and glass metallic (Ag-AgCl/Pt) Eh electrodes are used to determine pH and Eh of the microcosms.

5.3.3 Dissolved Oxygen. A dissolved oxygen meter with two polarographic electrodes is used to measure diel oxygen changes. The instrument should permit DO readings to 0.1 mg/L.

5.3.4 Heterotrophic Activity. A liquid scintillation counter calibrated for ^{14}C counting is required for measuring heterotrophic activity. An instrument with an automatic sample changer and hard copy printout is recommended

5.3.5 Microscopic Examinations. A good quality microscope is required to identify organisms in the microcosm inoculum. While species counts are not a monitored parameter, assurance that specific functional groups are present is required.

5.3.6 Microbial Agent Enumeration. Required equipment will vary depending on the agent tested and the methods of enumeration.

6. REAGENTS AND MATERIALS.

6.1 Media

6.1.1 Water Quality. All water used in preparing media and replacing evaporative loss from microcosm systems should be distilled and deionized.

6.1.2 Stock solutions. The chemically defined medium is a modification of Taub #82 (Taub and Read, 1982). Stock solutions are prepared and stored at 4° C.

Stock Solution	Compound	g/L Stock	mL Stock/L Medium
A	$NaNO_3$	8.5	5
B	$MgSO_4.7\ H_2)$	24.65	1
C	KH_2PO_4 NaOH	13.6 3.2	1
D	$CaCl_2$ or $CaCl_2.2H_2O$	11.1 14.7	20
E	NaCl	5.84	30
F*	EDTA NaOH $FeSO_4.7H_2O$	26.1 10.7 24.9	
G*	EDTA NaOH	29.0 12.0	
H*	H_3BO_3 $ZnSO_4.7H_2O$ $MnCl_2.4H_2O$ $Na_2MoO_4.2H_2O$ $CuSO_4.5H_2O$ $Co(N)_3)_2.6H_2O$	1.854 0.287 1.98 0.024 0.049 0.291	
IA	$AL_2(SO_4)_3;18H_2O$	3.2	1
IB	$Na_2SiO_3.9H_2O$	4.55	5

* Combined to make stock solution J, consisting of:

25 mL. F stock solution
50 mL. H stock solution
6 mL. G stock solution
19 mL. distilled, deionized water

This stock (J) is added to the final medium at the rate of .05 mL per liter.

900 mL of medium are required for each microcosm beaker.

6.1.3 Media preparation. At the time microcosms are started the appropriate proportions of stock solutions and distilled water are thoroughly mixed in a large polypropylene container. When mixing is complete, the medium will be quite alkaline and must be neutralized to pH 7.0 with 10 percent HCl.

Ideally, the mixing container should be large enough to hold the entire volume of media required. If this is not possible, the media should be mixed in two separate batches, and 450 mL of each batch added to each microcosm. This will ensure that the media composition is identical in all microcosms.

6.2 Sediment

6.2.1 Type. Washed, fine-grained sand is used as a sediment. It contains no nutient matter, and is used as a substrate for growth and a refuge for organisms. Beach sand is acceptable if it is clean and not mixed with organic matter.

6.2.2 Preparation. Each microcosm requires 50 mL of sand. The total amount of sand required for a test is placed in a container, covered with 10 percent HCl and mixed. After two hours, the acid is decanted and the sand flooded with distilled water. The water is mixed with the sand and poured off. This rinsing process is repeated until the rinse water reaches a pH of approximately 7.0. After rinsing the sand is oven dried at 75° C.

6.3 Stock Aquaria

6.3.1 Size. Stock communities of the organisms used in the test are maintained in 38 liter all glass aquaria. The aquaria may be held at room temperature and should be illuminated by a bank of fluorescent lights. A sheet of glass may be used to partially cover the top of the tank and retard evaporation.

6.3.2 Media. The stock communities are raised in T82, the same medium as used in the microcosms. The bottom of the stock tanks should be covered to a depth of approximately 10 cm with acid-washed sand.

6.4 Cleaning solutions. The stock aquaria and all microcosm vessels are cleaned according to the procedures suggested by Struempler (1973) and Leffler (1982). These procedures require laboratory detergent, 200 ,g/L hypochlorite solution and 10 percent HCl (see section 10.1.2).

7. SAFETY PRECAUTIONS.

7.1 Use of Cleaning Solutions. The solutions used in cleaning the vessels should be handled with caution. Rubber gloves and goggles should be worn during the cleaning process.

7.2 Avoiding Release of Test Organism. Of major importance in performing these tests is the use of proper aseptic technique. In addition to the recognized practices associated with the handling and transfer of microorganisms the following precautions should be taken.

7.2.1 Covering of test vessels. Microcosms are covered with petri dish lids except during sampling. This minimizes the possibility of airborne transfer of the test microorganisms.

7.2.2 Sanitizing monitoring probes. All monitoring probes should be sanitized after use. The sanitizing procedure of choice will depend on the microbe being tested.

7.2.3 Proper disposal of vessels and petri dishes. All used microcosm vessels, petri dishes, stirring rods and other glassware which come in contact with the tested agent microorganism should be autoclaved prior to disposal or cleaning and reuse.

8. MICROBIAL AGENTS.

To determine minimum effect densities and minimum survival densities, the microorganism tested should be inoculated into test microcosms

at a range of cell densitites.

8.1 Determining Inoculation Densities. Determination of the cell densities will be governed to some extent by the organism being tested. If initial tests at maximum hazard cell densities indicate no effects and no survival of the organism, further tests at lower concentrations are probably not required. If either effects or survival are seen at maximum concentrations, further tests should be performed to determine minimum effect and survival densities.

9. TEST ORGANISMS

9.1 Establishing the stock tank community.

9.1.1 Aquaria specifications. Stock cultures for inoculating microcosms are maintained in 38 L all-glass aquaria. These tanks may be kept in the laboratory at room temperature. Several banks of "cool white" fluorescent lights suspended over the tanks are controlled by a 12 hr light-12 dark time switch.

9.1.2 Preparing the stock culture. To prepare a stock culture, the tank is first thoroughly cleaned and rinsed (6.4). Washed sand (6.2.2) should be added to a depth of 1 - 2 cm and the tank filled with approximately 36 L of microcosm medium (6.1.2). Samples collected from natural ecosystems (approximately 2 L) should then be added to the aquarium.

At least one new stock culture should be prepared every six months to ensure a healthy, growing system. Inoculum from older stock cultures in addition to new "wild" material should be used to start a new culture.

A stock culture may be used for several years unless obvious changes suddenly occur in the the system. Periodic additions of

nutrient medium will aid in maintianing a healthy culture. In preparing inoculum for microcosms samples from several different aged stock cultures should be mixed together. No stock culture less than three months old should be used as a source of microcosm inoculum.

Distilled water is added to the stock cultures as needed to replace evaporative losses.

9.1.3 Source of organisms. Samples taken from natural ecosystems are used to start the stock cultures. A variety of system should be sampled in order to ensure a diversity of species in the stock cultures. Suitable sources include small ponds, marshes, vernal pools or other standing water sources.

9.1.4 Required functional groups. The following minimum criteria must be met in terms of species composition in the stock cultures. The diversity will most likely be far greater than this minimum.

(a) two species of single-celled green algae or diatoms.

(b) one species of filamentous green alga.

(c) one species of nitrogen-fixing blue-green alga.

(d) one grazing macroinvertebrate.

(e) one benthic, detritus-feeding macroinvertebrate.

(f) bacteria and protozoa species.

9.2 Evaluation of stock tank cultures. Stock tank cultures should be examined periodically to verify the presence of the required functional groups. Any groups that are missing should be added either by taking samples from other stock tanks or collecting fresh natural material.

If algae in the stock tanks appear to be in poor condition (e.g.

showing shrunken chloroplasts or reduced chlorophyll content) additional nutrient medium should be added.

9.3 Minimum time requirement for "co-adaption." The stock communities are allowed to mature for at least three months prior to use as a source of inoculum for the microcosms. This development period allows a relatively stable species assemblage to develop. Those species which are unable to coexist with other members of the community disappear, leaving behind a "co-adapted" group of species.

10. PROCEDURE.

10.1 Setup

10.1.1 Size of experiment - Number of microcosms. The number of treatment groups will depend on the number of cell concentrations to be tested. A typical chemical test requires 30 microcosms. These are divided into seven treatment groups (a control and five test groups) each containing five replicates. To ensure that the group of 30 is uniform at the time of treatment, a larger group of 35 microcosms is started and culled to 30 (see 10.4).

10.1.2 Vessel preparation. Microcosm vessels are washed five times with tap water, five times with 200 mg/L hypochlorite solution, five times with 10 percent HCl, and five times with distilled water. The cleaned vessels are kept inverted in a dust-free environment until needed.

10.1.3 Sediment. Each microcosm receives 50 mL of acid-washed, neutralized and oven dried (see 6.2.2) sand.

10.1.4 Media. Each microcosm recieves 900 mL of neutralized T82 medium (see 6.1.2).

10.1.5 Inoculation. The stock culture aquaria are stirred gently to bring

all particulate matter into suspension. A 1 L beaker is used to remove aliquots from each stock culture. These aliquots are combined in a 6 L polypropylene container. Transfers are handled gently and never poured through the air because of injury to the organisms.

Prior to inoculation, verify the presence of each of the required functional groups in this mixed stock culture. Following this, microscopic counts, using a Palmer counting slide (Wetzel and Likens, 1984), should be made to determine the algal density in the mixed stock. The algal density should be approximately 10^4 cells per mL. If necessary, adjust the inoculum to this concentration with either distilled water or aliquots of algae from more densely populated stock aquaria.

A stir bar is added to the 6 L container which is placed on a stir plate. Stirring is just sufficient to maintain particulates in suspension without injuring the organisms.

A 50 mL beaker is used to remove 50 mL aliquots from the 6 L container. These are carefully transferred to each beaker containing 50 mL of sand and 900 mL of medium. Again care is taken in the transfer not to injure the organisms.

Each microcosm is assigned a number which is marked on the outer surface of the beaker.

The microcosms are placed in the environmental chamber such that no two replicates of the same treatment are located on the same shelf. This creates a randomized block design.

10.1.6 Cross-seeding. Twice weekly for three weeks following inoculation all microcosm are cross-seeded.

Each microcosm is stirred gently and a 50 mL beaker is used to remove a 50 mL from the 6 L container and return them gently to each microcosm.

10.1.7 Reinoculation. Throughout the life of the microcosm, each is reinoculated weekly using the procedure outlined. 10.1.6. Following the weekly reinoculation distilled water is added to each microcosm to return its volume to 1000 mL.

10.2 Measurements. Ecosystem level effects are measured through periodic monitoring of the variables listed below. It is important that all measurements be made at at the same time of day during each sampling period. The following results must be reported for each microcosm:

(a) pH value to 0.1 pH units

(b) pE value to 1 millivolt

(c) Rate of oxygen gain 0.01 $mgO_2/L/hr$.

(d) Rate of oxygen loss 0.01 $mgO_2/L/hr$.

(e) Heterotrophic Activity in dpm/15mL/2 hr.

(f) Population density of the microbial agent.

If the growth chamber lights are set to come on a 8 AM and off at 8 PM, the morning measurements should be started at approximately 6:30 AM, or early enough to finish before the lights come on. Afternoon measurements can be started at approximately 4:30 PM, while the lights are still on. The light regime must be a 12 hr-on 12 hr-off schedule, but the on-off times can be shifted from 8 AM and 8 PM. If such shifts are made, sampling times should be adjusted accordingly.

10.2.1 Dissolved Oxygen. Oxygen gain and loss are estimated by the three-point diel dissolved oxygen method (McConnell, 1962; Abbott, 1966;

Leffler, 1978; and Hendrix et al., 1981). Measurements are scheduled such that morning and afternoon readings are taken on the first day of a sampling period, and a second morning reading is taken on the following day. The dissolved oxygen electrode must be standardized against air-saturated water before each sampling session. The procedures are given by the American Public Health Association (1975).

To measure oxygen levels in the microcosms, the oxygen electrode is inserted into the center of the microcosm and gently stirred until the readings stabilize. A separate electrode should be used for the control group (10.6.2).

The electrodes should be thoroughly rinsed with distilled water before being moved from one microcosm to another.

10.2.2 pH and Redox potential (Eh). Before each sampling session, the pH and Eh electrodes should be standardized. A separate set of electrodes must be used for the control group.

Phosphate buffer of pH 7 is suitable for calibrating the pH electrode.

The glass metallic combination (Ag-AgCl/Pt) Eh electrode may be standardized against the solution described by Light (1972). Both of these standards are of relatively high ionic strength and tend to slow the response of the electrodes to conditions in the relatively low ionic strength of the microcosms. Accordingly, both electrodes should be held in an extra microcosm until readings stabilize before beginning the actual sampling.

A glass combination pH electrode is inserted into the microcosm such that its tip is in the center of the beaker halfway between the bottom and the surface. The microcosm is not stirred. The

electrode is allowed to equilibrate, usually 3-5 minutes, before the reading is taken. Otherwise the procedures follow standard practice (American Public Health Association, 1975).

The Eh electrode is inserted into the microcosm such that its tip is in the center of the beaker halfway between the bottom and the surface. The microcosm is not stirred. Readings usually stabilize in about 15 seconds.

Since Eh is influenced by pH, Eh values should be corrected to standard pH 7 (Wetzel, 1983, p. 299) by the relationship E7 (mv) = Eh (mv) + 58 (pH - 7). The electrodes should be thoroughly rinsed with distilled water before being moved from one microcosm to another.

10.2.3 Heterotrophic Activity. Heterotrophic Activity is measured by the ^{14}C method described by Wright and Hobbie (1966) and Thomas (1985). Sampling is achieved by placing the microcosm on a stir plate. A stir bar is inserted and rotated at approximately 60 rpm. A wide mouth pipet is inserted so that its tip is halfway between the center of the beaker and the wall and halfway between the bottom and the surface. A 15 mL sample is withdrawn. If sampling occurs on reinoculation days, it should precede the reinoculation procedure.

A 0.3 mL aliquot of ^{14}C-glucose (0.15 μCi) is added to a 15 mL sample from each microcosm. After a 15 minute incubation period, the sample is acidified and incubated for two hours during which CO_2 is trapped on a strip of filter paper saturated with a CO_2 absorbing agent. The ^{14}C radioactivity of the paper is then counted by liquid scintillation.

The stir bar and pipet should be thoroughly rinsed with distilled water when they are moved from one microcosm to another.

10.2.4 Population density of MPCA or GEM. Measurement of survival of the microbial agent is critical. The enumeration system will vary with the organism being evaluated so specific procedures cannot be given here.

10.3 Pre-treatment monitoring. Microcosms should be sampled twice during the six week development phase. All variables (10.2) should be measured in each microcosm on day 31 and day 38 of the pre-treatment period.

In addition, well stirred samples should be withdrawn from each microcosm and examined microscopically to verify the presence of the required functional groups.

10.4 Culling Procedure. To improve replicability, the initial group of microcosms must meet a set of quality control criteria. Microcosms failing to meet these criteria or varying significantly from the rest of the group are culled before treatment.

10.4.1 Failure to achieve minimum quality control standards. Microcosms failing to meet the minimum quality control cirteria (Sec. 11) for successful development are removed and not used in the test.

10.4.2 Calculation of group means. After removal of any microcosm for failure to meet minimum criteria, any remaining extra systems are culled by calculating the group mean for each of the ecosystem-level variables measured. Those microcosms showing the greatest deviation from these means are culled until the remaining group is reduced to the desired size (usually 35).

The remaining set of systems is randomly assigned numbers, and

arranged into treatment groups of five replicate microcosms. The remaining set of systems is randomly assigned numbers, and arranged into treatment groups of five replicate microcosms.

10.5 Treatment

10.5.1 Technique for adding toxic agent. The method of adding the test organism will vary depending on its formulation. It may be added in dry form or diluted in a series of sterile blanks. If media dilution blanks are used, an equivalent amount of sterile media must be added to each contol microcosm.

10.6 Post-treatment monitoring.

10.6.1 Schedule. Microcosms are normally monitored for six weeks following the additon of the test organism. For the first two weeks all ecosystem-level variables (Sec. 10.2) are measured twice a week (i.e., on days 2, 4, 9, and 11) with the exception of heterotrophic activity which is measured once each week. Thereafter, all variables are measured once a week on days 18, 25, 32, and 39. If no deviations from controls are observed in any treatment being tested for a two week period the monitoring may be terminated prior to the end of six weeks.

Determinations of the survival of the microbial agent should be made once a week on a schedule corresponding to that of the heterotrophic activity measurements.

10.6.2 Use of separate set of control and treatment electrodes. To avoid contamination of the controls with the microbial agent, a separate set of electrodes should be used for all oxygen, pH, and Eh measurements on controls.

10.6.3 Sterilization of equipment. Waste disposal. Liquid wastes generated

through test organism additions and ^{14}C methodology must be collected and disposed in accordance with EPA, NIH, and NRC regulations.

Contaminated solid wastes such as biomass, filters, charcoal, ion exchange resin, vermiculite, glassware, gloves, etc., must be packaged and disposed in accordance with EPA and NRC regulations.

11. QUALITY ASSURANCE.

To assure that a test has been successful, the following criteria must be met:

11.1 Pre-treatment criteria. All functional groups should be present in the systems at the last pre-treatment measurement.

By the end of the development period the morning dissolved oxygen levels should not fall below 4.0 mg/L and evening dissolved oxygen levels should reach at least 11 mg/L.

11.2 Post-treatment criteria. If two control microcosms become significantly different from the rest of the control group, for any variable, that control should be discarded and not used for any calculations.

If the microbial agent appears in two or more of the controls the experiment should be aborted.

If the microbial agent appears in one of the controls, that system should be discarded and not used in any calculations.

12. DATA ANALYSIS.

12.1 Determination of minimum effect. If the microbial agent was tested at several initial cell densities and at least one of the treament groups showed no significant effect a minimum effect or no effect concentration should be estimated.

12.1.1 Dunnett's procedure. Dunnett's procedure (Steel and Torrie, 1960) is used to determine which, if any of the treatments are different from the control ($p <= 0.5$). This test should be performed on each variable measured for each sampling day. This permits a determination of which variables are significantly affected and the duration of the effect.

These calculations may be summarized by plotting the difference (or deviation) of each treatment group mean from the control mean. In these figures the control mean appears as a straight line centered at zero. An area corresponding to the upper an lower bounds of Dunnett's significant difference ($p < .05$) is plotted around the zero line (control mean) and defines a region where the various treatments are not significantly different from the control. Points lying outside the shaded area are significantly different ($p < .05$) from the control.

From these calculations, the minimum and no-effect concentrations of the microbial agent are determined.

12.2 Determination of Organism Survival. From the periodic counts of the density of the microbial agent, determinations of the survival rate of the organism can be reported.

References

Abbott, W. 1966. Microcosm studies on estuarine waters. I. The replicability of microcosms. J. Water Poll. Cont. Fed. 39:258-270.

American Public Health Association. 1975. Standard Methods for the Examination of Water and Wastewater. American Public Health Assoc., Washington, DC, 1193 pp.

Betz, F., M. Levin, and M. Rogul. 1983. Safety aspects of genetically-engineered microbial pesticides. Rec. DNA Tech. Bull. 6:4 pp 135-141.

Environmental Protection Agency. 1979. Toxic Substance Control Act: Premanufacture testing of new chemical substances. Federal Register, March 16, 19079, pp. 16240-16292.

Environmental Protection Agency. 1985. Freshwater Aquatic Invertebrate Toxicity and Pathogenicity Testing: Tier 1 In FIFRA Regulations, Subdivision M, Section B: Microbial Testing. Washington, DC.

Hendrix, P.F., C.L. Langer, E.P.Odum, and C.L. Thomas. 1981. Microcosms as Test Systems for the Ecological Effects of Toxic Substances: An Appraisal with Cadmium. Final Report of EPA Grant No. R805860010. U.S.E.P.A. Environmental Research Laboratory, Athens, Georgia.

Leffler, J.W. 1978. Ecosystem response to stress in aquatic microcosms. In Energy and Environmental Stress in Aquatic Systems. J.H. Thorp and J.W. Gibbons (eds.), DOE Symposium Series, Augusta, GA, Nov. 2-4, 1977. National Technical Information Service, Springfield, VA.

Leffler, J.W. 1982. Microcosms and Stress Criteria for Assessing Environmental Impact of Organic Chemicals. Final Report, Battelle Columbus Laboratories, Columbus, Ohio.

Mallory, Larry M., Chang-Soo Yuk, Li-Nuo Liang, and Martin Alexander. 1983. Alternative prey: A mechanism for elimination of bacterial species by protozoa. Appl. and Envr. Micro. 46:5 pp. 1073-1079.

McConnell, W.J. 1962. Productivity relations in carboy microcosms. Limnol. and Oceanogr. 7:336-343.

National Academy of Sciences. 1981. Testing for Effects of Chemicals on Ecosystems. A Report by the Committee to Review Methods for Ecotoxicology. National Academy Press, Washington, DC, 98 pp.

Schindler, J.E., J.B. Waide, M.C. Waldron, J.J. Hains, S.P. Schreiner, M.L. Freedman, S.L. Benz, D.R. Pettigrew, L.A. Schissel, and P.J. Clark. 1980. A microcosm approach to the study of biogeochemical systems. 1. Theoretical rationale. In Microcosms in Ecological Research, J.P. Giesy (ed.), DOE Symposium Series, Nov. 8-10, 1978. Augusta, GA, National Technical Information Service, Springfield, VA.

Schneider, R.A. 1981. Classes of ecotoxicological tests: their advantage and disadvantages for regulation. In Working Papers Prepared as Background for Testing for Effects of Chemicals on Ecosystems. National Academy of Sciences, National Academy Press, Washington, DC.

Steel and Torrie. 1960. Principles and Procedures of Statistics McGraw-Hill New York. 481 pp.

Stotsky, G. and H. Babich. 1984. Fate of genetically-engineered microbes in natural environments. Recom. DNA Tech. Bull. 7:4 pp 163-188.

Struempler, A.W. 1973. Adsorption characteristics of silver, lead, cadmium, zinc, and nickel on borosilicate glass, polyethylene, and polypropylene container surfaces. Anal. Chemistry. 45:2251-2254.

Taub, F.B. and P. Read. 1982. Standardized Aquatic Microcosm Protocol. Final Report, Vol. II. FDA Contract 223-80-2352.

Thomas, C.L. 1985. Development of a Test System for Screening Toxic Substances; A Comparison Using Organic Substances. Ph.D. Dissertation. Virginia Polytechnic Institute and State University, Blacksburg, VA. 183 pp.

Wetzel, R.G. 1983. Limnology. Saunders College Publishing. Philadelphia, PA. 767 pp.

Wetzel, R.G. and G.E. Likens. 1984. Limnological Analyses. W.B. Saunders Co. Philadelphia, PA. 357 pp.

Wright, R.T. and J.E. Hobbie. 1966. The use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology. 47:447-464.

METHODS FOR ESTABLISHING MICROCOSMS OF AQUATIC LITTORAL ECOSYSTEMS FOR DETERMINING SAFE LEVELS OF AGENT EXPOSURE[1,2]

by

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1. SCOPE.

This protocol describes procedures for establishing freshwater littoral communities in a laboratory with materials collected from natural ponds. The methods were developed to assess effects of chemical toxicants on aquatic communities (4), however, the systems may be adaptable to testing the potential impacts of microbial pesticdes on freshwater ecosystems.

2. APPLICABLE DOCUMENTS.

2.1 1976 Annual Book of ASTM Standards, Part 31:

D888	Oxygen, dissolved in water
D1067	Acidity and Alkalinity
D1125	Conductivity
D1126	Hardness
D1293	pH
D1426	Ammonia

3. SUMMARY.

Littoral ecosystems are established in 72 L glass aquaria using sediments, water, and biota from natural ponds. The microcosms are easily replicated and require little maintenance. These microcosms are static systems (no inflow or outflow) and retain a functional similarity to natural ponds for more than 6 months under artificial lighting. In testing for effects of toxicants, at least five chemical concentrations, a no-treatment control, and a solvent control (if a carrier solvent is used) are recommended with replication. Chemical (pH, hardness, conductivity, and dissolved oxygen) and biological (e.g., zooplankton and bacterial densities, and chlorophyll concentrations) are made periodically throughout the experimental period. No-observable-effect-concentrations (NOEC) are determined using Dunnett's procedure.

4. SIGNIFICANCE.

Single-species laboratory tests are useful for measuring the relative toxicity of chemical pollutants, but *a priori* they cannot predict effects that may occur in natural environments. The microcosms described here closely resemble, both structurally and functionally, the ecosystems from which they are derived. Responses of these systesm to chemical toxicants and microbial pesticides are likely to mimic those of natural communities. Such microcosms have been used in parallel experiments with outdoor ponds to evaluate effects of organic contaminants on an aquatic community (6). The results from these experiments confirm that the microcosms may be used as surrogates for ponds in toxicity tests.

The microcosms are easy to assemble, require minimal maintenance, are replicable, and are stable for periods of at least 6 months. Because the microcosms are established from natural ecosystems, the nature of the microcosm communities will vary with the source. This allows for site-specific evaluation of toxicant effects.

5. DEFINITION OF TERMS.

5.1 Agent - chemical or microbial toxicant for whose effects on aquatic ecosystems are being measured.

5.2 Littoral - describing shallow-water habitats that are transitional between terrestrial and deep-water habitats.

5.3 Net Primary Production - a measure of the net amount of carbon fixed by the community. In the system described here, the carbon fixation rate is estimated from the net rate of oxygen production (oxygen produced - oxygen consumed), allowing for oxygen diffusion

into or out of the microcosm.

5.4 Overnight Respiration - the amount of oxygen consumed by the community during the 12 h dark period, allowing for oxygen diffusion. In this protocol, the lengths of dark and light periods are equal and overnight respiration is assumed to be equal to daytime respiration.

5.5 Ecosystem Metabolism - a measure of the energy balance within the community. It is estimated based on the ratio of net primary production (P) to overnight respiration (R). If P:R is greater than 1.0, the system is autotrophic (fixes more energy than it consumes). If P:R is less than 1.0, the system is heterotrophic (consumes more energy than it fixes). Ecosystem metabolism exerts a strong influence upon chemical conditions within the system.

6. APPARATUS.

6.1 Facilities. Microcosms may be set up in either an environmental chamber or a laboratory area with restricted traffic, to minimize disturbance. Temperature should be kept relatively constant since the systems will respond to rapid temperature fluctuations.

6.2 Lighting. Wide-spectrum fluorescent lamps are used to supply 150 to 250 $\mu E\ m^2\ s^{-1}$ (1050 to 1900 foot-candles) of photosynthetically active radiation (400 to 700 nm) with a 12-h daily photoperiod. As is shown in Figure 1, nine lamps (e.g., Westinghouse Colortone 50, high output) will provide sufficient light for ten microcosms, with minimal hotspotting.

6.3 Aquaria. The microcosms are contained in 72-L glass aquaria (40 cm deep x 60 cm long x 30 cm wide). (Smaller microcosms are difficult to replicate; larger ones take up too much space.) Before use, aquaria and all other glassware are cleaned using a

nonphosphate detergent and are rinsed successively with dilute HCl, saturated Na_2CO_3, and distilled water.

6.4 Sampling Tubes. Glass tubes, about 40 cm long and with an inner diameter of 2 cm, are used to collect water samples.

6.5 Delivery System. Chemical toxicant solutions can be introduced into the microcosms using an adjustable volumetric pipette. A delivery system will be developed for each microbial agent.

7. MATERIALS.

7.1 Sediment. Sediment is collected from the upper 10 cm of the bottom of a natural pond. In shallow ponds, the sediment may be collectd using a shovel. In deeper ponds, an Ekman dredge can be used. It is best to collect sediments when ambient water temperatures are within 10°C of the temperture at which the experiment will be conducted to avoid exposing biota to temperature shock.

7.2 Water. Water is collected into clean plastic carboys from an undisturbed area of the pond in the same general location from which the sediment is taken.

7.3 Plants. Macrophytes and/or filamentous algae are collected from the same area of the pond from which sediment and water were taken. Care should be taken to prevent overexposing of the plants to sunlight.

7.4 Agents. Chemical toxicants should be of reagent grade. Solutions of the toxicant should be made using distilled water or reagent-grade solvents. For microbial agents, the strains tested should be of the same degree of purity and activity as those for whose release is anticipated.

8. PRECAUTIONS.

8.1 Some chemical and microbial agents have the potential to affect humans adversely if precautions are not taken. Therefore, contact with all these materials should be minimized. Information on toxicity to humans and recommended handling procedures should be studied before tests are begun.

8.2 Care should be taken to ensure that large crustaceans, (e.g., crayfish) are excluded from the microcosms. The burrowing activities of these animals will severely disrupt the microcosms.

9. TEST ORGANISMS.

9.1 The organisms that would comprise a microcosm community depend upon the source of materials. The dominant organisms in microcosms described in reference 3 were *Elodea canadensis* (macrophyte); *Spirogyra sp.*, *Gonium* sp., *Euglena* sp., and *Phacus* sp. (algae); *Alona costata*, *Chydorus sphaericus*, and *Simocephalus vetulus* (cladocerans); and *Cyclops* spp., *Eucyclops agilis*, and *Macrocyclops albidus* (copepods).

10. PROCEDURES.

10.1 Microcosm Construction. Up to twenty microcosms can be assembled by two persons in about 8 h. Sediment is collected and sieved to remove large debris and stones. The sediment is distributed equally among the aquaria until each has at least a 5-cm layer (~10 L per microcosm). The aquaria are filled with pond water to within 2 cm of the top. The water should be added so as to prevent undue disturbance of the sediment. A simple device, such as an inverted beaker or a plastic bottle with holes punched around the bottom, can be used to dissipate the force of the water. If sufficient care is taken, any suspended sediment will settle

overnight. Once the water column is clear, about 100 g (drained weight) of macrophyte or algae is put into each aquarium, and a portion of each plant is gently pushed into the sediment to anchor it in place. All other biota enter the microcosm along with the sediment, water, or plants.

About 7 to 14 d are required for the microcosms to reach an equilibrium. Cross-inoculation during this pretreatment period improves replicability among microcosms.

10.2 Temperature. The temperature range selected will depend upon the communites and conditions being modeled. Caution should be taken to avoid rapid fluctuations in temperature that might perturb the microcosms.

10.3 Experimental Design. To measure ecosystem responses to chronic exposure and determine safe levels of chemical toxicants, it is necessary to adequately define an exposure-response curve. Standard practice is to use a geometric series of five exposure levels and a no-treatment control (if a carrier solvent is used for the toxicant, a solvent control should also be tested). Each exposure level is replicated at least once, preferably two or more times. The microcosms are monitored during all phases of the experiment: pretreatment, to determine attainment of equilibrium; treatment, to measure responses and post-treatment, to evaluate recovery.

10.4 Application of the Agent. For chemicals, the toxicant is dissolved either in water or an organic solvent (usually methanol). Appropriate volumes of toxicant solution are injected below the water surface, using an adjustable volumetric pipette, according to a predetermined schedule. The pipette is moved around the microcosm during the

application to promote distribution of the toxicant. Methods of applying microbial pesticides will be developed for each agent.

10.5 Water Chemistry. Conductivity and water temperature are measured *in situ* three times a week, using commercially available portable instruments. Samples integrating chemical conditions throughout the water column are collected by vertically immersing a glass sampling tube to within a few centimeters of the sediment. The top of the tube is sealed with a stopper and the tube and its contents are lifted out of the microcosm. Samples taken from two or three locations within the microcosm are sufficient to characterize the water chemistry. Hardness, ammonia, pH, and other parameters are measured from such samples twice weekly.

10.6 Dissolved Oxygen. Dissolved oxygen (DO) is measured using a DO meter twice daily: at its peak concentration (at the end of the light period) and its lowest concentration (at the end of the dark period).

10.7 Zooplankton samples. Zooplankton are collected by rapidly submerging a 2-L beaker into a microcosm. The zooplankton are concentrated in a Wisconsin-type plankton bucket equipped with 80 micron mesh, stained with methylene blue, narcotized with Neo-Synephrine hydrochloride, and preserved in sucrose-formaldehyde solution. Zooplankton are counted and identified to genus or species. Whole samples are counted unless debris or high zooplankton density make subsampling necessary.

10.8 Planktonic Bacteria. Once a week 0.2 to 0.5 mL aliquots of 100 mL samples are stained with acridine-orange fluorescent dye and filtered through 0.4 um polycarbonate filters. Filters are examined by epifluorescent microscopy (2). Bacteria in twenty 2500-um^2 fields are counted.

11. CALCULATIONS.

11.1 Ecosystem metabolism is estimated from diurnal changes in DO (5). Net primary production (P) and overnight respiration (R) are calculated by:

$$P = A - 12\ (D) \text{ and } R = B + 12\ (D)$$

where A is the increase in DO during the day, B is the decrease in DO at night, and D = the rate of oxygen diffusion into the microcosms (units for A, B, P, and R are mg oxygen per L water per hr). The rate of oxygen diffusion is related to the percentage saturation and can be estimated by measuring the rate at which DO concentration increases in an aquarium filled with sterile, oxygen-depleted water.

11.2 Weekly means of parameters at each toxicant concentration are compared with control means using Dunnett's method (1). Toxicant concentrations at which responses are not significantly different from control values are NOECs.

12. QUALITY ASSURANCE.

Because determination of toxicant effects is based on comparison of treated microcosms and controls, it is necessary to establish stable control conditions with as little variance as possible among control replicates. Several factors impact the replicability of microcosms and care should be taken to consider these during the conduct of the experiment. Collection, transportation, and distribution of water, sediment, and biota should be done in such a manner as to minimize stress on plants and animals. Even distribution of collected materials during set-up and cross-innoculation during the pretreatment period will help reduce variability among microcosms.

Adequate and uniform lighting will promote development of similar macrophyte communities. Because these microcosms are sensitive to rapid fluctuations in temperature, ambient air temperature should be kept to within 3°C of the selected experimental temperature.

13. REPORT

The results reported should include the following:

13.1 Name of the test, investigator, laboratory, and test date.

13.2 A brief description of the toxicant or agent, including its source and lot number or other identifying features.

13.3 A description of the source of the sediment, water, and biota; the methods of collection; and the date materials were collected.

13.4 A detailed description of the communities that develop in the microcosms, including a list of the dominant species in each and any variations among microcosms.

13.5 A description of the significant or observable responses at each treatment level, including the NOEC.

REFERENCES

Chew, V. 1977. Comparisons Among Treatment Means in an Analysis of Variance. U.S. Department of Agriculture, Agriculture Research Service, ARS/H/6, Washington, DC.

Daley, R.J. 1977. Direct Epifluorescence Enumeration of Native Aquatic Bacteria: Uses, Limitations, and Comparative Accuracy. In Native Aquatic Bacteria: Enumeration, Activity, and Ecology, J.W. Costerton and R.R. Colwell, Eds., ASTM STP 695, American Society for Testing and Materials, Philadephia, PA, pp. 29-45.

Franco, P.J. et al. 1984. Effects of Chronic Exposure to Coal-derived Oil on Freshwater Ecosystems: I. Microcosms. Environ. Toxicol. Chem. 3:447-463.

Giddings, J.M. 1985. A Microcosm Procedure For Determining Safe Levels of Chemical Exposure in Shallow-Water Communities. Presented at the Symposium on Community Toxicity Testing, Colorado Springs, Colorado, May 6-7, 1985.

Giddings, J.M. and G.K. Eddlemon. 1978. Photosynthesis/Respiration Ratios in Aquatic Microcosms Under Arsenic Stress. Water Air Soil Pollut. 9:207-212.

Giddings, J.M. and P.J. Franco. 1985. Calibration of Laboratory Bioassays with Results from Microcosms and Ponds. In Validation and Predictability of Laboratory Methods for Assessing the Fate and Effects of Contaminants in Aquatic Ecosystems, T.P. Boyle, Ed., ASTM STP 865, American Society for Testing and Materials, Philadelphia, PA, pp. 104-119.

TERRESTRIAL SYSTEMS

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"ENCLOSED SYSTEMS FOR TESTING MICROBIAL PEST CONTROL AGENTS"

by

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INTRODUCTION

The United States Environmental Protection Agency's Office of Pesticide Programs is responsible for providing registrants with protocols regarding risk assessment issues for microbial pest control agents (MPCAs). This responsibility includes the new generation of MPCAs, microbes modified by recombinant DNA or other technologies to produce genetically modified microbial pesticides (GMMPs).

Enclosed multispecies test systems could offer a convenient means to conduct exposure assessments in habitat-simulating fashion eliminating the need for environmental release of GMMPs during the test evaluation period. Some of the challenges in establishing protocols for the enclosed multispecies test systems include the very limited database on effects research involving GMMPs, the lack of appropriate bioassays for measuring possible effects from GMMPs, and the modest experiences in maintaining multispecies in enclosed test systems.

Virtually all that is available in the literature concerning multi-species tests comes from toxicity evaluations involving the effects of man-made chemicals on nontarget organisms. Information supplied by the participants of this working group strongly reflects this literature database. Information is provided on commercially available biota for possible inclusion into an enclosed system and a considerable amount of information is provided concerning the design and application characteristics of one of the many terrestrial microcosms which have been described in the literature. The greatest challenge of this working group has been to determine the selection of nontarget species, the number and population size to include in any enclosed test system, how those species may be

maintained and for how long, and a lack of data to determine appropriate end-points for measuring effects from MPCAs and GMMPs on nontarget species and ecosystems.

ENCLOSED TEST SYSTEMS

A terrestrial microcosm can be defined as a confined portion of a terrestrial ecosystem, subject to controls, that can be used to investigate and evaluate ecological processes (Van Voris, et al., 1985). Terrestrial microcosms provide flexibility to the investigator in permitting the control of numerous variables relevant to the determination of risk assessment issues. Microcosms are more likely to reflect natural ecosystem responses then most single species bioassays (Tolle, Arthur, and Van Voris, 1983). Single species assays will not reveal potential perturbations in population densities when certain predators, competitors, or ecosystem functional activities are altered. For populations of several species to exist in enclosed terrestrial microcosms, suitable trophic levels and certain habitat simulating features must be maintained. If predatory insects are enclosed for example, a suitable and consistent supply of prey, of course, must be included in the system. This could complicate the experimental microcosm if unusual nutritional requirements arise in maintaining one or more predator/prey combinations. Furthermore, one should expect fluctuations in predator/prey numbers as a function of each species density, stage of growth, growth rate and conditions under which the microcosm is maintained. How changes in beneficial prey cell numbers can be specifically related to harm caused by the GMMPs will be a very difficult issue to resolve since there are many factors which influence their number.

Terrestrial microcosms come in numerous shapes and designs each

offering the unique creativity of the designer and the novel purpose(s) for which it is to be used (see Figs 1-9; Gillett, Witt, and Watt, 1979). Discussions of this working group did not consider in any detail the diversity of existing terrestrial microcosms or microcosm technologies. Discussions of the Working Group centered predominantly on the Experimental Terrestrial Soil-Core microcosm (TSCM) developed at Battelle, NW Laboratory (Fig. 9). The advantages of this terrestrial microcosm are summarized in the following items.

1. The TSCM has been adopted as a standard by the American Society for Testing and Materials and is an EPA/OTS terrestrial microcosm test protocol for evaluating toxicity of chemicals (Van Voris, 1985; Van Voris, Tolle, and Arthur, 1985).

2. The TSCM is adaptable in terms of sizes of the intact soil core, the number of soil core units which simultaneously can be replicated, and in the enclosure design which uses HEPA filters.

3. The experimental design involves the incorporation of an undisturbed soil core which maintains the natural biological and physical stratification of the soil(Van Voris et al., 1985).

4. The TSCM can be used to evaluate a number of parameters relevant to the risk assessment of GMMPs including: a) an evaluation of fate and survival characteristics; b) genetic stability; and c) effects on nontarget species and possible influences on various ecological processes such as niche displacement and microbial functional activities such as nutrient cycling.

The containment aspects of the TSCM have been anticipated in the design which involves a Lexan® wall enclosure and a quartz glass top with a stainless steel air-tight lower compartment to enclose the soil and

microcosm tubes. The entire enclosed system is to be maintained under slight negative air pressure and all air passing through the system will be double HEPA filtered prior to exhaust. Two HEPA filters of laminar flow grades with 99.97% and 99.99% efficiency would be installed in sequence to insure some 8-log reduction in particles of 0.3 um and larger from moving out of the enclosure. Since virus particles are of the order of 0.002-0.05 um in size, an additional feature provides for installation of an incinerator on the exhaust side of the microcosm to insure that smaller particles would not escape.

Additional features of the enclosed TSCM include the use of glove box type access ports and an ethylene oxide gas flush sample transfer chamber (Fig. 9). Since the enclosed TSCM is on wheels it can be readily moved between various environmental chambers. As designed, the TSCM meets the BL-3 containment level guideline of the National Institutes of Health for research with recombinant DNA. The estimated cost for construction of two complete enclosed TSCM units is about $34,000. The dimensions of such a unit are approximately 12 ft long, 4 ft high with about 32 cubic ft of space within the enclosure to accommodate 12 soil core units. The size of the individual cores is approximately 60cm deep and 20cm in diameter. The depth is sufficient to allow for the establishment of deep-rooted plants.

BIOTA FOR MICROCOSMS

A. Insects

Many resource materials were provided to the working group including a listing of 20 groups of beneficial arthropods which might be included in a terrestrial line missing details). Information provided includes a listing of commercial insectaries, source materials, as well as methods

for rearing both the beneficial arthropods and, as appropriate, the corresponding prey or host species of the beneficial arthropod. The original list of 20 beneficial arthropods provided in Appendix A was reduced here to those that are most readily available and are easier to propagate or maintain. A list which contains only a single representative from each taxonomic group from Appendix A is summarized in Table 2.

Table 2. Systems of beneficial arthropods suggested for inclusion in risk assessment testing of microbial pesticides.

SYSTEM	BENEFICIAL SPECIES	COMMENTS
#1	Amblyseius hibisci	a beneficial mite; 11 day life-cycle at 25C
#4	Geocoris punctipes	big-eyed bug; a variety of food will suffice including cabbage looper; 34 day life-cycle at 26.7C
#5	Chrysoperla carnea	green lacewing; 24-27 day life-cycle at 26.7C
#7	Olla abduminalis	apparently only available by field collection; 15 day life-cycle at 26C
#9	Trichogramma sp.	parasite of insect eggs, primarily lepidopterans 8-10 day life-cycle at 26.7C
#13	Lysiphlebus testaceipes	parasite of the greenbug; not active below 18.3C; 13 day life-cycle at 21C
#14	Cotesia melanoscelus	parasite of gypsy moth; 15 day life-cycle at 23C
#15	Metaphycus helvolus	parasite of black scale 13 day life-cycle at 27.8C
#16	Aphytis lingnanensis	parasite of California red scale; 17 day life-cycle at 26C

Table 2. (Cont'd)

#17	Brachymeria intermedia	parasite of lepidopterans 15-30 day life-cycle at 24C
#18	Lixophaga diatraeae	fly parasite on sugarcane borer; USDA mass produces; 18-20 day life-cycle at 26C
#19	Apis mellifera-	honeybee; freshly emerged bees from a brood comb should live the longest in confinement (perhaps 30 days)
#20	Vanessa cardui	The only butterfly considered for tests;feeds on over 100 plant species; life of about 7 weeks

B. Plants and invertebrates.

The following list of plants and other invertebrates represents those species commonly employed in enclosed microcosm test systems. The species are readily available from various sources such as seed companies and biological supply houses (See Appendix B).

Table 3. Plants suggested for inclusion in risk assessment testing of microbial pesticides.

1. alfalfa	6. winter wheat	10. corn
2. clover	7. soybean	11. cotton
3. oats	8. tomato	12. trifolium
4. ryegrass	9. pine and Douglas fir seedlings	
5. timothy		

Table 4. Other invertebrates and non-beneficial insects suggested for inclusion in risk assessment testing of microbial pesticides.

1. cricket	3. earthworm	5. pillbug
2. nematode	4. collembola	6. millipede

Sources of the above biota are given in many of the references appended and a noninclusive list of supply organizations is provided in Appendix B.

FACTORS TO CONSIDER IN THE DESIGN AND EXECUTION OF MICROCOSM TESTS.

In establishing the risk assessment of a GMMP, the working group sees the need for duplicate enclosed microcosms. Both microcosms would receive suitable nontarget species as well as the target (pest) species of the GMMP. Only one microcosm would receive the GMMP itself. Possible effects in the negative control (no GMMP) would be compared in severity and number to those recorded in the experimental microcosm. One can gauge the relative merits of the test by ascertaining rates of population decline in the negative control microcosm. One may also use population declines in the negative control to assess the level of species compatibility and general"health" of the microcosm.

The materials used in the TSCM should reasonably reflect those at the site destined to receive the GMMP in the small scale open field test. Thus, the soil core should be taken from this field site along with a representative amount of natural vegetation.

The lists of flora and fauna previously listed above are intended to provide flexibility to the agency program offices regarding selection and requirements of further nontarget species. The number of different species included in the microcosm tests should probably not exceed 5 to 7 added test species. The workgroup recommends that similar representatives from the list of flora and fauna should be suggested to all registrants for enclosed test trials for purposes of continuity of test evaluations and consistency in agency requirements. The list of test species added to the microcosm may include, as examples; a grass, broadleaf and cereal grain (e.g., ryegrass, alfalfa, wheat), cricket, and earthworm as well as two or three species from the list of beneficial arthropods. In addition to the indigenous plant species taken with the soil core, these recommended

species should provide a reasonable measure of species diversity for nontarget tests. Higher species of organisms such as avian and mammals cannot be satisfactorily maintained in this type of enclosed microcosm. The possible effects of GMMPs on these species will be evaluated in single species tests.

Inclusion in the microcosm of the intended target species of the GMMP is strongly recommended by this working group. The target species can serve as an internal control to verify the efficacy of the GMMP risk assessment by making sure sufficient GMMP numbers were added to result in an effective dose. The presence of infected target species will also provide localized high concentrations of the GMMP which can therefore be more "naturally" evaluated for ecological fate and hazards. If a predetermined population of target species is not eliminated, then the enclosed microcosm risk assessment experiment is probably invalid.

The kinds of evaluations and duration of the exposure tests in the enclosed microcosm will be influenced by the nature and number of the species introduced into the enclosure. Thus, life-cycles of most beneficial arthropods are greatly influenced by temperature and will be completed within 3 to 4 weeks for most species. However, the three to four week life-cycle should not be considered as a short-coming of the microcosm evaluation system. The completion of life-cycles with sexual maturity and egg laying, could become a part of the end-points selected for assessment of risks.

Choices of stocking densities of target and nontarget species represent a challenge. Thus, stocking densities of plants may have physical constraints and may be based upon common agricultural practices. However, insufficient experiences do not allow more than best "estimates" for

stocking densities of other species. In the 20 cm X 60 cm soil core, it should be possible to maintain 4 to 8 earthworms for several weeks, without significant disturbance of the ecosystem. On the other hand, densities of suitable prey/host numbers will largely determine the population density of beneficial predator/parasite that can be supported. In a space of 32 cubic ft perhaps 50 specimens of each beneficial arthropod can be maintained if only two or three species are used. Obviously prey or host specimens must also be provided. This will necessitate adding to the microcosm suitable nurse plants or other food sources for supporting and maintaining viable prey or host specimens.

It seems most likely that the major benefit of conducting enclosed testing will be the information gained on the ecological hazards as opposed to hazards involving toxicity, virulence, and pathogenicity effects on non-target species. The microcosm will provide a realistic measure for testing the recovery of the GMMP from natural habitat material. Furthermore, it will also be possible to gain information on the fate, survival, ecological competitiveness, and genetic stability of the test agent. The specific procedures for conducting these ecological risk assessments are not available. However, identification, detection, and fate analyses can be conducted by most microbial ecologists if appropriate genetic tags and selective media are used to facilitate the detection of the GMMP.

Specific recommendations for recovering microbes from plant, insect, and invertebrate tissues, from soil, and the use of appropriate buffers, blending conditions, etc. are beyond the scope of this document. Some of these particulars will necessitate research and all will certainly require trial and error efforts to optimize recovery. Nevertheless, flora and

fauna will be analyzed qualitatively and quantitatively for the GMMP. These analyses should be conducted until two successive attempts to recover the GMMP are negative. If the GMMP is found in association with nontarget specimens, the biological nature of the association must be determined. Thus, histopathology and/or electron microscopy coupled with suitable means to locate and identify the GMMP must be conducted. Evidence should be provided so that it is obvious beyond any reasonable doubt that the association of the GMMP with a nontarget species is transient and is of no biological significance.

Measurement of effects of GMMPs on other microbes, microbial processes, and activities is a current topic of research now being supported by this agency. However, there are certain assays that can be conducted to assess ecological risks and EPA should consider these in evaluating risks of new GMMPs. The TSCM is designed so that fluid leachate from soil cores can be collected. Thus, GMMP cell densities and environmental fate and survival including mobility through soil can be quantitatively measured in these leachates. A basic investigation of the total microbial profile in leachates collected from the experimental and control microcosms should be made. A total bacterial plate count should be reported as well as estimates of numbers of different colony types appearing on a standard medium. A similar evaluation should be conducted for fungal populations. These measurements can be used to evaluate impact of the GMMP on microbial species diversity and population size.

Various mineral analyses can also be conducted on the leachate. Some comparison of leachate chemical quality and quantity in the experimental system must be made with the negative control microcosm which did not receive the GMMP. Specific analyses of the leachate should involve

measurement of the solution pH plus organic carbon, total carbon, ammonia, nitrate, and inorganic phosphate. Replication of analyses involving leachates from different soil cores is appropriate.

Bibliography

Ausmus, B. S., D. R. Jackson, and P. Van Voris. 1979. The accuracy of screening techniques. In: J. M. Witt and J. W. Gillett, eds. Symposium on Terrestrial Microcosms and Environmental Chemistry, June, 1977, Corvallis, OR. NSF/RA 79-0026.

Beall, M. L., Jr., R. G. Nash, and P. C. Kearney. 1976. Agroecosystem--a laboratory model ecosystem to simulate agricultural field conditions for monitoring pesticides. In: Environmental Modeling and Simulation, W. Ott, ed. EPA-600/9-76-016. U.S. Environmental Protection Agency, Washington, D. C. pp. 790-793.

Bezark, L.G., and H. Yee. 1985. Suppliers of beneficial organisms. Calif. Dept. Food and Ag. Biological Control Services Program. Sacramento, CA. 95832. pp. 1-6.

Cole, L. K., R. L. Metcalf, and J. R. Sanborn. 1976. Environmental fate of insecticides in a terrestrial model ecosystem. Intern. J. Environ. Studies. 10:7-14.

Draggan, S. 1976. The microprobe analysis of 60-Cobalt uptake in sand microcosms. Xenobiotica 6:557-563.

Gile, J. D., J. C. Collins, and J. W. Gillett. 1982. Fate and impact of wood preservatives in a terrestrial microcosm. J. Agric. Food Chem. 30:295-301.

Gile, J. D. 1983. 2,4-D--Its distribution and effects in a ryegrass ecosystem. J. Environ. Qual. 12:406-412.

Gillett, J. W., and J. D. Gile. 1976. Pesticide fate in terrestrial laboratory ecosystems. Intern. Environ. Studies. 10:15-22.

Gillett, J. W., J. M. Witt, and C. J. Watt (eds). 1979. Symposium on Terrestrial Microcosms and Environmental Chemistry. June, 1977, Corvallis, OR. NSF/RA 79-0026.

Lichtenstein, E. P., T. W. Fuhrmann, and K. R. Schulz. 1974. Translocation and metabolism of 14C-phorate as affected by percolating in a model soil-plant ecosystem. J. Agric. Food Chem. 22:991-996.

Lichtenstein, E. P., K. R. Schultz, and R. R. Liang. 1977. Fate of fresh and aged soil residues of the insecticide 14C-N-2596 in a soil-corn-water ecosystem. J. Econ. Entomol. 70:169-175.

Lighthart, B., H. Bond, and B. G. Volk. 1977. The use of soil/litter microcosms with and without added pollutants to study certain components of the decomposer community. In: J. M. Witt and J. W. Gillett, eds. Symposium on Terrestrial Microcosms and Environmental Chemistry, June, 1977, Corvallis, Or. NSF/RA 79-0026.

Metcalf, R. L., G. K. Sangha, I. P. Kapoor. 1971. Model ecosystem for the evaluation of pesticide degradability and ecological magnification. Environ. Sci. Technol. 5:709-713.

Tolle, D. A., M. A. Arthur, and P. Van Voris. 1983. Microcosm/field comparison of trace element uptake in crops grown in fly ash-amended soil. Sci. Total Environ. 31:242-261.

Van Voris, P. 1985. Standard guide for terrestrial soil-core microcosm. ASTM Test Procedure. Draft for E47.08 subcommittee.

Van Voris, P., D. A. Tolle, and M. F. Arthur. 1985. Experimental terrestrial soil-core microcosm test protocol. PB85-213338, EPA/600/3-85/047. U.S. Environmental Protection Agency, Corvallis, OR. 97333.

Van Voris, P., D. A. Tolle, M. F. Arthur, and J. Chesson. 1985. Terrestrial microcosms: applications, validations, and cost-benefit analysis. In: J. Cairns, Jr., (ed.). Multispecies toxicity testing, Pergamon Press, New York. pp 117-142.

APPENDIX A

Included in Appendix A is a recommended list of beneficial arthropods for enclosed systems testing. Provided with this material is information for insect propagation, life-cycle characteristics, a list of suitable prey/hosts, source materials and relevant literature citations. Also included is a copy of the pamphlet, "Suppliers of Beneficial Organisms in North America", published by the State of California, Department of Food and Agriculture.

In the last section of the Appendix A is a copy of the Standard Guide for Terrestrial Soil-Core Microcosm, that was submitted to ASTM by Dr. Van Voris.

Proposed Non-Target Terrestrial Organisms for Testing Microbial Pest Control Agents (L.K. Etzel)

rthropod-Plant Systems	References Related to Rearing	Sources for Starting Colonies	Notes
Predaceous Mites			
A. Acarina:Phytoseiidae			
(1) *Amblyseius hibisci* on *Tetranychus urticae* (2-spotted mite) on lima bean plants	Kennett & Hamai, 1980	CDFA list; EntSoc list	*A. hibisci* easily reared on pollen (oak, magnolia, & cattail pollens good)
(2) *Metaseiulus occidentalis* on *T. urticae* on lima bean plants	Ditto	Ditto	
(3) *Phytoseiulus persimilis* on *T. urticae* on lima bean plants	Ditto	Ditto	
Insect Predators			
A. Heteroptera:Lygaeidae			
(4) *Geocoris punctipes* on noctuid-moth eggs on corn plants	Cohen, 1981 Cohen & Debolt, 1983	EntSoc list; Cohen (?)	Common in North America. Can be reared on artificial diet.
B. Neuroptera:Chrysopidae			
(5) *Chrysoperla carnea* (green lacewing) on *Acyrthosiphon pisum* (pea aphid) on fava bean plants or alfalfa	Morrison, 1985a (for *C. carnea*) Forbes et al.,1985 (for aphids)	CDFA list; EntSoc list	
. Coleoptera:Coccinellidae			
(6) *Hippodamia convergens* on *A. pisum* on fava bean plants or alfalfa	Hagen & Sluss, 1966 (for *Hippodamia*) Forbes et al., 1985 (for aphids)	CDFA list; EntSoc list (for aphid)	

Arthropod-Plant Systems	References Related to Rearing	Sources for Starting Colonies	Notes
(7) Olla abdominalis on A. pisum on fava bean plants or alfalfa		Field collection; EntSoc list (for aphid)	Common arboreal species in North America. Artificial diet soon to be developed.
(8) Cryptolaemus montrouzieri on Pseudococcidae (mealybugs) on citrus trees or oleanders or potato sprouts	Fisher, 1963	CDFA list; EntSoc list	
II. Insect Parasites			
A. Hymenoptera:Trichogrammatidae			
(9) Trichogramma spp. on Ostrinia nubilalis (Eur. corn borer) eggs or Heliothis zea (corn earworm) eggs on corn plants	Burbutis & Goldstein, 1983 Morrison, 1985b	CDFA list; EntSoc list	
B. Hymenoptera:Mymaridae			
(10) Anaphes flavipes on Oulema melanopus (cereal leaf beetle) eggs on cereal grain plants	Anderson, 1968	EntSoc list	
C. Hymenoptera:Aphidiidae			
(11) Aphidius ervi or smithi on A. pisum on fava bean plants or alfalfa	UC-Berkeley (unpubl.) Forbes, et al.,1985 (for aphids)	EntSoc List	
(12) Diaretiella rapae on Myzus persicae (green peach aphid) or Brevicorynae brassicae (cabbage aphid) on Chinese cabbage or kale	Simpson et al., 1975	Field collection; EntSoc list (for aphids)	Common parasite in North America.

Arthropod-Plant Systems	References Related to Rearing	Sources for Starting Colonies	Notes
(13) *Lysiphlebus testaceipes* on *Schizaphis graminum* (greenbug) on oat plants	Starks & Burton, 1977	CDFA list; EntSoc list	
D. Hymenoptera:Braconidae			
(14) *Cotesia* (=*Apanteles*) *melanoscelus* on *Lymantria dispar* (Gypsy Moth)	Chianese, 1985 (for *C. melanoscelus*) ODell et al., 1985 (for *L. dispar*)	EntSoc list	
E. Hymenoptera:Encyrtidae			
(15) *Metaphycus helvolus* on *Saissetia oleae* (black scale) on oleander	Flanders, 1942	CDFA list	
F. Hymenoptera:Aphelinidae			
(16) *Aphytis melinus* (or *A. lingnanensis*) on *Aspidiotis nerii* (oleander scale) on butternut pumpkins (*Cucurbita moschata*)	DeBach & White, 1960 Papacek & Smith, 1985	EntSoc list; Field collection (for oleander scale)	
G. Hymenoptera:Chalcididae			
(17) *Brachymeria intermedia* on *Trichoplusia ni* (cabbage looper) on cabbage or other suitable plant	Palmer, 1985 (for *B. intermedia*) King & Hartley, 1985a (for *Galleria mellonella*) Guy et al., 1985 (for *T. ni*)	EntSoc list	Attacks several lepidopterous species. Can be easily raised on *G. mellonella* (the greater wax moth)
H. Diptera:Tachinidae			
(18) *Lixophaga diatraeae* on *Diatraea saccharalis* (sugarcane borer) on plants (?)	King & Hartley, 1985b (for *L. diatraeae*) King & Hartley, 1985c (for *D. saccharalis*)	EntSoc list	

Arthropod-Plant Systems	References Related to Rearing	Sources for Starting Colonies	Notes
III. Other Beneficial or Innocuous Insects			
A. Hymenoptera:Apidae			
(19) *Apis mellifera* (honeybee) in cage with honey, water, and plants (alfalfa or mustard)		Apiary; Carolina Biol. Supply Co.; Wards Natural Science Establishment, Inc.	
B. Lepidoptera:Nymphalidae			
(20) *Vanessa cardui* (painted lady butterfly) on host plants	Morton, 1979 Dietz, etal., 1976	Carolina Biol. Supply Co.; Wards Natural Science Establishment, Inc.	The most widely distributed butterfly. Hosts include burdock, thistles, sunflowers, nettles. The references listed are for artificial diets given in Singh (1985).

Proposed Non-Target Terrestrial Organisms
for Testing Microbial Pest Control Agents (L.K. Etzel)

I. General References

1. Bezark, L.G. and H. Yee
1985. 1985 Suppliers of Beneficial Organisms in North America. California Department of Food and Agriculture, Biological Control Services Program, 3288 Meadowview Road, Sacramento, CA 95832. 6 pp.

2. Dickerson, W.A., J.D. Hoffman, E.G. King, N.C. Leppla, and T.M. ODell
1979. Arthropod Species in Culture in the United States and Other Countries. Entomological Society of America, College Park, MD. 93 pp.

3. Singh, P. and R.F. Moore (eds.)
1985. Handbook of Insect Rearing. Volumes I and II. Elsevier, New York. 488 pp. & 514 pp.

II. Specific References

4. Anderson, B.C.
1968. The biology and ecology of Anaphes flavipes, an exotic egg parasite of the cereal leaf beetle. Ph.D. Thesis. Purdue University, 148 pp.

5. Burbutis, P.P. and L.F. Goldstein
1983. Mass rearing Trichogramma nubilale on European corn borer, its natural host. Protection Ecology, 5:269-275.

6. Chianese, R.
1985. Cotesia melanoscelus. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 395-400, Elsevier, New York, 488 pp.

7. Cohen, A.C.
1981. An artificial diet for Geocoris punctipes (Say). Southwest. Entomol., 6:109-113.

8. Cohen, A.C. and J.W. Debolt
1983. Rearing Geocoris punctipes on insect eggs. Southwest. Entomol., 8:61-64.

9. DeBach, P. and E.B. White
1960. Commercial mass culture of the California red scale parasite Aphytis lingnanensis. Calif. Agric. Exp. Stn. Bull. No. 770, 58 pp.

10. Deitz, L.L., J.W. Van Duyn, J.R. Bradley, Jr., R.L. Rabb, W.M. Brooks, and R.E. Stinner
1976. A guide to the identification and biology of soybean arthropods in North Carolina. Agric. Exp. Sta. Tech. Bull. 238, 264 pp.

11. Fisher, T.W.
1963. Mass culture of Cryptolaemus and Leptomastix, natural enemies of citrus mealybug. Calif. Agr. Expt. Stn. Bull., 797:39.

12. Flanders, S.E.
1942. Metaphycus helvolus, an encyrtid parasite of the black scale. Jour. Econ. Entomol., 35:690-698.

13. Forbes, A.R., B.D. Frazer, and C.K. Chan
1985. Aphids. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 353-359. Elsevier, New York, 488 pp.

14. Guy, R.H., N.C. Leppla, and J.R. Rye
1985. Trichoplusia ni. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 487-494. Elsevier, New York, 514 pp.

15. Hagen, K.S. and R.R. Sluss
1966. Quantity of aphids required for reproduction by Hippodamia spp. in the laboratory. Pp. 47-59. In "Ecology of Aphidophagous Insects" (I. Hodek, ed.), Dr. W. Junk, Publ., The Hague, Netherlands, 360 pp.

16. Kennett, C.E. and J. Hamai
1980. Oviposition and development in predaceous mites fed with artificial and natural diets (Acari:Phytoseiidae). Ent. Exp. Appl., 28:116-122.

17. King, E.G. and G.G. Hartley
1985a. Galleria mellonella. In "Handbook of Insect Rearing" (P.Singh and R.F. Moore, eds.), Vol. II, pp. 301-305, Elsevier, New York, 514 pp.

18. King, E.G. and G.G. Hartley
1985b. Lixophaga diatraeae. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 119-123, Elsevier, New York, 514 pp.

19. King, E.G. and G.G. Hartley
1985c. Diatraea saccharalis. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 265-270, Elsevier, New York, 514 pp.

20. Morrison, R.K.
1985a. Chrysopa carnea. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 419-426, Elsevier, New York, 488 pp.

21. Morrison, R.K.
1985b. Trichogramma spp. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 413-417, Elsevier, New York, 488 pp.

22. Morton, A.C.
1979. Rearing butterflies on artificial diets. J. Res. Lepid., 18:221-227.

23. ODell, T.M., C.A. Butt, and A.W. Bridgeforth
1985. Lymantria dispar. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 355-367, Elsevier, New York, 514 pp.

24. Palmer, D.J.
1985. Brachymeria intermedia. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 383-393, Elsevier, New York, 488 pp.

25. Papacek, D.F. and D. Smith
1985. Aphytis lingnanensis. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 373-381, Elsevier, New York, 488 pp.

26. Simpson, B.A., W.A. Shands, and G.W. Simpson
1975. Mass rearing of the parasites Praon sp. and Diaretiella rapae. Ann. Entomol. Soc. Am., 68:257-260.

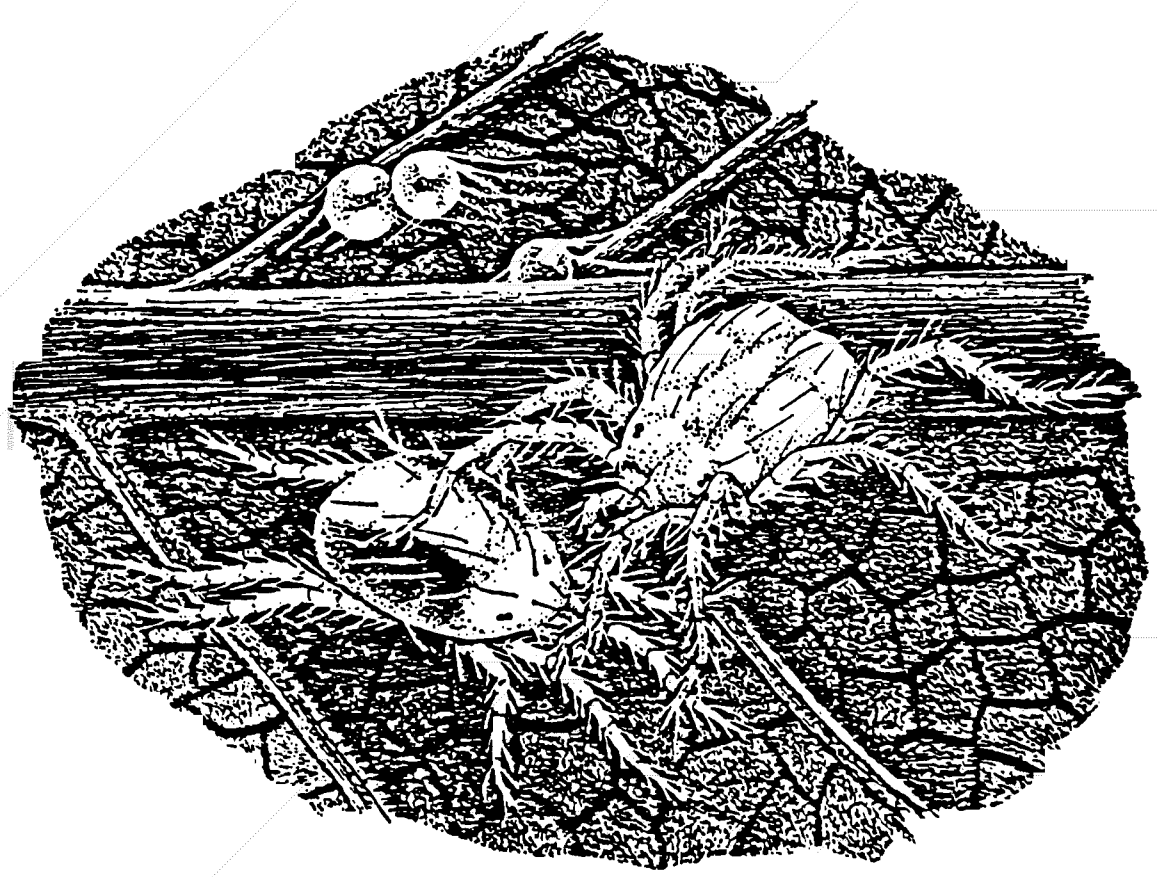
27. Singh, P.
1985. Multiple-species rearing diets. In "Handbook of Insect Rearing" (P. Singh & R.F. Moore, eds.), Vol. I, pp. 19-44, Elsevier, New York, 488 pp.

28. Starks, K.J. and R.L. Burton
1977. Greenbugs: determining biotypes. USDA-ARS Tech. Bull., 1556:12.

State of California
Department of Food and Agriculture

1985
SUPPLIERS OF BENEFICIAL ORGANISMS
IN NORTH AMERICA

Larry G. Bezark
Helen Yee
Biological Control Services Program
Division of Pest Management, Environmental Protection and Worker Safety



"A beneficial mite attacking a pest mite"

Artwork by Linda Heath

Insects, mites, snails and diseases, although usually thought of as undesirable, can be very useful for controlling various pests. Biological control is the use of beneficial organisms for pest control. These beneficial organisms can be predators such as ladybugs, lacewings and praying mantids, which feed on other insects. Others, such as nematodes and wasps including *Trichogramma*, are parasites. *Trichogramma* wasps lay their eggs in the eggs of caterpillar pests, where they develop by feeding on the pest's egg. Some beneficial insects attack noxious weeds. Fungi, bacteria and viruses can also be used to control insect and weed pests.

The 1982 version of this list has been sent in response to thousands of requests, to individuals, universities, extension entomologists, and many others. The list has appeared in part or in its entirety, in several magazines and has been mailed out separately as part of a larger publication on resources for organic pest control. Since this list was last published three years ago, we have added 23 new suppliers. In addition, there are 13 organisms now available that were not for sale in 1982.

Green Lace-Wings	Lady-Bugs	Praying Mantids	Egg Wasps	Other Organisms (see last page)	Supplier	Mail Order	Phone Order	Retail	Whole-sale	No
				CGP	Ag Bio Chem Inc. 3 Fleetwood Ct. Orinda, CA 94563 (415) 254-0789	X	X		X	Free Brochu
				GWP, PMP	Applied Bionomics P.O. Box 2637 Sidney, B.C. Canada V8L 4C1 (604) 656-2123	X	X	X	X	Free Catalog Need Permit For US Import
				MD, RSM	Associates Insectary P.O. Box 969 Santa Paula, CA 93060 (805) 933-1301	X	X	X	X	
				FPG, FPS FPZ, MB	Beneficial Biosystems 1603-F 63rd Street Emeryville, CA 94608 (415) 655-3928	X	X	X		Whole-sale For PCA's
				FPM, FPN FPS, GHP	Beneficial Insectary 245 Oak Run Road Oak Run, CA 96069 (916) 472-3715	X	X	X	X	Postage Paid
	X				Beneficial Insects Company P.O. Box 556 Brownsville, CA 95919 (916) 675-2251	X	X	X	X	Free Brochur
X			X	FP	Beneficial Insects Ltd. P.O. Box 154 Banta, CA 95304	X		X		Lacewing April-Jul
				APA, GWP PMM, PMP	Better Yield Insects 13310 Riverside Drive E. Tecumseh, Ontario Canada N8N 1B2 (519) 735-0002	X	X	X	X	Need per For US Import
				DS	Bio-Con Systems P.O. Box 377 Sunnymead, CA 92388 (714) 656-1712	X	X	X	X	Free Brochure
X	X	X	X	FP	Bio-Control Company P.O. Box 247 Cedar Ridge, CA 95924 (916) 272-1997 Ron Rogers	X	X	X	X	Free Brochure
X			X	PMA	Biogenesis, Inc. P.O. Box 36 Mathis, TX 78368 (512) 547-3259	X	X	X	X	
X	X		X	BSP, FPM, FPS GHP, GWP, LT, MD NPV, PMC, PMO PMP, RSM	Bio Insect Control 710 S. Columbia Plainview, TX 79072 (806) 293-5861	X	X	X	X	
				PMC, PML PMO, PMP	Biotactics, Inc. 7765 Lakeside Drive Riverside, CA 92509 (714) 685-7681 after 6 pm	X	X	X	X	Free Brochure

Green Lace-wings	Lady-Bugs	Praying Mantids	Egg Wasps	Other Organisms	Supplier	Mail Order	Phone Order	Retail	Whole-sale	Notes
				PN, PNB PNC, PNG	B.R. Supply Company P.O. Box 845 Exeter, CA 93221 (209) 732-3422	X	X	X	X	
				BP	Burgess Seed & Plant Co. Department 69 905 Four Seasons Road Bloomington, IL 61701 (309) 663-9551	X	X	X		Free Catalog
	X	X	X		Burpee Seed Company 300 Park Avenue Warminster, PA 18974	X				Free Catalog
X			X	FP, NOW NOWP	California Green Lacewings P.O. Box 2495 Merced, CA 95340 (209) 722-4985	X	X	X	X	Free Literature & Price List
			X	GHP, PNC	Colorado Insectary P.O. Box 3266 Durango, CO 81302 (303) 247-5360	X	X	X	X	Free Brochure
				DS	Decollate Snails P.O. Box 972 Lake Arrowhead, CA 92352 (714) 337-2282 after 7 pm	X	X	X	X	Free Brochure Send SASE
				BP	Fairfax Biological Laboratories, Inc. Clinton Corners, NY 12514 (914) 266-3705	X	X	X	X	Free Brochure
				BP, GHP MB	Farmers Seed and Nursery Department 72 2207 East Oakland Avenue Bloomington, IL 61701 (309) 663-9551	X	X	X		Free Catalog
				DS	Fillmore Decollate Snail Farm 2841 West Young Road Fillmore, CA 93015 (805) 524-1030 (714) 781-7643	X	X	X	X	Free Brochure
	X		X	BSP, DS, FP MD, RSM	Foothill Ag. Research Inc. 510 W. Chase Drive Corona, CA 91720 (714) 371-0120	X	X	X	X	Free Literature
	X				Fountain's Sierra Bug Co. P.O. Box 114 Rough & Ready, CA 95975 (916) 273-0513	X	X	X	X	Free Brochure
			X		Gothard Inc. P.O. Box 7 Mesilla, NM 88046 (505) 522-9031	X	X	X	X	Free Brochure
X	X	X	X	FP, GHP	Gurney Seed & Nursery Co. Yankton, SD 57079 (605) 665-4451	X	X	X		Free Catalog

Green Lace-Wings	Lady-Bugs	Praying Mantids	Egg Wasps	Other Organisms	Supplier	Mail Order	Phone Order	Retail	Whole sale	Notes
X	X		X	DS,FPM,FPP FPR,FPS,GHP GWP,MB,MD,NPV PMC,PML,PMO PMP,RSB,RSM	Harmony Farm Supply P.O. Box 451 Graton, CA 95444 (707) 823-9125	X	X	X	X	Free Catalog
X	X	X	X	BP, FP GHP, MB	Henry Field's Seed and Nursery Company Shenandoah, IA 51602 (712) 246-1888	X	X	X		Free Catalog
X	X		X	BSP,CMD,DS FPM,FPS,FPT GHP,GWP,MD,PMC PMO,PMP,RSM	Integrated Pest Management 305 Agostino Road San Gabriel, CA 91776 (818) 287-1101	X	X	X	X	Some Literature
X	X	X	X	BP, FP GWP, MD PM	King's Natural Pest Control 224 Yost Avenue Spring City, PA 19475	X		X	X	Postpaid Free Literature
X	X		X	FP, FPS PMA	Kunafin Trichogramma Insectaries Route 1, P.O. Box 39 Quemado, TX 78877 (512) 757-1468 (512) 773-0149	X	X	X	X	Free Information
X	X	X		FP	Lakeland Nurseries Sales Inc. 340 Poplar Street Hanover, PA 17331 (717) 637-5555	X	X	X		Free Catalog
X	X	X	X	BP, FPS, FPZ GHP, GWP, MCP MD	Mellinger's Nursery 2310 W. South Range Road North Lima, OH 44452 (216) 549-9861	X	X	X		Free Catalog
				BP	Miller Nurseries 5060 West Lake Road Canandaigua, NY 14424	X		X		Free Catalog
				DS	Chuck Musgrove Riverside, CA (714) 780-8733		X		X	Will not Ship
		X			Nationwide Seed & Supply 4801 Fengenbush Lane Louisville, KY 40228	X				Postage Paid
X	X	X	X	BSP, FPM, FPN FPS, GWP, MB MD, MF, PBP, PMC, PML, PMO PMP, RSM	Natural Pest Controls 8864 Little Creek Drive Orangevale, CA 95662 (916) 726-0855	X		X	X	Free Brochure
X	X			GWP, MD PMC, PML PMO, PMP	Nature's Control P.O. Box 35 Medford, OR 97501 (503) 773-5927	X	X	X	X	Free Brochure
X	X		X	BBP, BP, CPP FP, GHP, GWP, MB MD, NPV, PMC PMO, PMP, PNC	Necessary Trading Company P.O. Box 603 New Castle, VA 24127 (703) 864-5103	X	X	X	X	Charge for Catalog
				PN	The Nematode Farm, Inc. 3335 Birch Street Palo Alto, CA 94306 (415) 494-8630				X	

Green Lace-wings	Lady-Bugs	Praying Mantids	Egg Wasps	Other Organisms	Supplier	Mail Order	Phone Order	Retail	Whole sale	Notes
	X	X			Organic Control, Inc. 5132 Venice Blvd. Los Angeles, CA 90019	X		X	X	Free Catalog Postpaid
X	X		X	BSP, FP, GWP MB, MD, PMC, PML, PMO, PMP RSM	Organic Pest Control Naturally P.O. Box 55267 Seattle, WA 98155 (206) 367-0707	X		X	X	Charge for Catalog
				BP	Richard Owen Nursery Department 74 2300 East Lincoln Street Bloomington, IL 61701 (309) 663-9551	X	X	X		Free Catalog
				DS	Pacific Tree Farms 4301 Lynwood Drive Chula Vista, CA 92010 (619) 422-2400	X	X	X	X	Charge for Catalog
X	X	X	X	BP,BSP,DS,FPM FPS,GHP,GWP,MD MF,NOW,NPV,PMC PMH,PML,PMO,PMP PN,PNC,RSB,RSM	Peaceful Valley Farm Supply 11173 Peaceful Valley Road Nevada City, CA 95959 (916) 265-3276	X	X	X	X	Free Catalog
				BP, GHP, MB	Reuter Laboratories P.O. Box 346 Haymarket, VA 22069 1-800-368-2244	X	X	X	X	Free Brochure
X	X	X	X	APA-Canada only FP-US only GWP-Canada only PMP-Canada only	Richters P.O. Box 26 Goodwood, Ontario Canada L0C 1A0 (416) 640-6677	X	X	X		Canada Orders Only in June Need permit For US Import
X	X		X	BSP, DS, FP GWP, MD, PMC PMO, PMP	Rincon-Vitova Insectaries, Inc. P.O. Box 95 Oak View, CA 93022 (805) 643-5407	X	X	X	X	Free Brochure Visa and MC
				BBP	Rocky Mountain Insectary P.O. Box 152 Palisade, CO 81526 (303) 245-0406	X	X	X	X	Free Brochure
			X	FPG, FPM, FPS GWP, MD	Spalding Laboratories 760 Printz Road Arroyo Grande, CA 93420 (805) 489-5946	X	X	X	X	Free Brochure
X	X	X	X	FP, GWP	Unique Insect Control P.O. Box 15376 Sacramento, CA 95851 (916) 967-7082	X	X	X	X	Free Literature
	X	X			West Coast Ladybug Sales P.O. Box 903 Gridley, CA 95948 (916) 534-0840	X	X	X	X	Free Literature
				PMO	Wilk, Kitayama and Mead 9093 Troxel Road Chico, CA 95928 (916) 895-8424 (M-W-F)		X	X	X	Sulfur & OP Resistant Strain

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GREEN LACEWINGS-*Chrysopa carnea*
LADY BUGS-*Hippodamia convergens*
PRAYING MANTIDS-*Tenodera aridifolia sinensis*
EGG WASPS-*Trichogramma minutum*
Trichogramma platneri
Trichogramma pretiosum

APA-Aphid parasite-*Aphidoletes aphidimyza*
BBP-Bean beetle parasite-*Pediobius foveolatus*
BP-Milky spore-*Bacillus popilliae*
BSP-Black scale parasite-*Metaphycus helvolus*
CGP-Crown gall preventive-*Agrobacterium radiobacter*
CMD-Citrus mealybug parasite-*Leptomastix dactylopii*
CPP-Colorado potato beetle parasite-*Edovum puttleri*
DS-Decollate snail-*Rumina decollata*
FP-Fly parasites-*various species*
FPG-Fly parasite-*Sphegigaster sp.*
FPM-Fly parasite-*Muscidifurax raptor*
FPN-Fly parasite-*Nasonia vitripennis*
FPP-Fly parasite-*Pachycrepoideus vindemiae*
FPR-Fly parasite-*Carcinops sp.*
FPS-Fly parasite-*Spalangia endius*
FPT-Fly parasite-*Sphegigaster tachinaephagus zealandicus*
FPZ-Fly parasite-*Muscidifurax zaraptor*

GPH-Grasshopper pathogen-*Nosema locustae*
GWP-Greenhouse whitefly parasite-*Encarsia formosa*
LT-Greenbug parasite-*Lysiphlebus testaceipes*
MB-Mosquito bacterium-*Bacillus thuringiensis israeliensis*
MCP-Mormon cricket pathogen
MD-Mealybug destroyer-*Cryptolaemus montrouzieri*
MF-Mosquitofish-*Gambusia sp.*
NOW-Navel orangeworm parasite-*Goniozus legneri*
NOWP-Navel orangeworm parasite-*Pentalitomastix sp.*
NPV-Nuclear polyhedrosis virus
PBP-Pink bollworm parasite-*Microchelonus blackburni*
PM-Predatory mites-*various species*
PMA-Predatory mite (for ants)-*Pyemotes tritici*
PMC-Predatory mite-*Amblyseius californicus*
PMH-Predatory mite-*Amblyseius hibisci*
PML-Predatory mite-*Phytoseiulus longipes*
PMM-Predatory mite-*Amblyseius mckenziei*
PMO-Predatory mite-*Metaseiulus occidentalis*
PMP-Predatory mite-*Phytoseiulus persimilis*
PN-Parasitic nematodes-*various species*
PNB-Parasitic nematode-*Neoaplectana bibionis*
PNC-Parasitic nematode-*Neoaplectana carpocapsae*
PNG-Parasitic nematode-*Neoaplectana glaseri*
RSB-Red scale parasite-*Comperiella bifasciata*
RSM-Red scale parasite-*Aphytis melinus*

The caterpillar larval bacterium *Bacillus thuringiensis* is available through many retail and wholesale concerns under various brand names.

Inclusion in this pamphlet does not imply an endorsement of the suppliers or their products by the California Department of Food and Agriculture, nor does it imply criticism of other suppliers not listed. This pamphlet may be freely reproduced. One copy per request is available from:

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May 17, 1985
Peter Van Voris
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Designation: EXXXX-85

AMERICAN SOCIETY FOR TESTING AND MATERIALS
1916 Race St., Philadelphia, PA 19103

Standard Guide for

TERRESTRIAL SOIL-CORE MICROCOSM

This standard is issued under the fixed designation EXXX; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of the last revision. A number in parentheses indicates the year of last approval. This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate, and is the direct responsibility of Subcommittee E47.08 on Biological Field Tests.

1.0 SCOPE

1.1 This standard guide defines the requirements and procedures for testing the environmental fate, ecological effects and environmental transport of chemicals in terrestrial ecosystems using the soil-core microcosm. Additionally, it provides a general rationale for the approach and materials suggested for use in the soil-core microcosm.

1.2 This test procedure is designed to supply site-specific or possibly regionalized information on the probable chemical fate and ecological effects resulting from release or spills of chemicals in the environment in either liquid or solid form.

1.3 Experience has shown that microcosms are best applied chemical in the assessment process at stages after aquisition of preliminary knowledge about a chemical's properties and biological activity. The data resulting from performance of this standard can then be used to compare the potential terrestrial environmental hazards of a chemical.

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1.4 *This standard guide may involve hazardous materials, chemicals, radiolabeled compounds, hazardous operations and wastes. This standard guide does not purport to address all of the safety problems associated with testing the environmental toxicity and environmental fate of a pure chemical or complex mixture of chemicals. It is the responsibility of whoever uses this standard to consult and establish the appropriate safety and health protocols and determine the applicability of regulatory limitations for wastes prior to performing the test.*

2. APPLICABLE DOCUMENTS

2.1 ASTM Standards:

D18 Test Methods for Soils and Rocks
(D422-63, D2167-84, D2216-80, and D2488-84)

D19 Test Methods for Water Quality Analysis
(D511-84, D515-82, D1254-67, D1426-79, and D3867-79)

2.2 Environmental Protection Agency 1982. Environmental Effects Test Guideline. Washington, DC. Office of Pesticides and Toxic Substances, U. S. EPA EPA 560/6-82-002. Available from NTIS, Springfield, VA PB82-232992.

2.3 Environmental Protection Agency. 1982. Environmental Effects Test Guideline. Washington, DC. Office of Pesticides and Toxic Substances, U.S. EPA EPA 560/6-82-002. Available from NTIS, Springfield, VA PB82-232992.

2.4 Environmental Protection Agency. 1982. Chemical Fate Test Guideline. Washington, DC. Office of Pesticides and Toxic Substances, U.S. EPA EPA 560/6-82-003. Available from NTIS, Springfield, VA PB82-233008.

3.0 DEFINITION OF TERMS

3.1 *Soil-Core Terrestrial Microcosm* - The terrestrial microcosm or micro-ecosystem is defined here as a physical model of an interacting

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community of autotrophs, omnivores, herbivores, carnivores and decomposers within an intact soil profile. Specifically, it is an intact soil-core containing the natural assemblages of biota surrounded by the boundary material. The system includes all equipment, facilities, and instrumentation necessary to maintain, monitor, and control the environment. The forcing functions, e.g., light intensity and duration, water quality and watering regime, temperature, and toxicant dose for the test system, are under the investigator's control. This test system is distinguished from test tube and single species toxicity tests by having a natural assemblage of organisms present, resulting in a higher order of ecological complexity and, thus, the capacity to evaluate chemical effects on component interactions and ecological processes. Certain features of this test system however, set limits on the types of questions that can be legitimately addressed. Those limitations are related to scale and sampling, which in turn constrain both (a) the type of ecosystems and species assemblages on which one can hope to gain information, and (b) the longevity of the test system.

3.2 *Physical, Chemical and Biological Conditions* - The physical, chemical, and biological conditions of the test system are determined by the type of ecosystem from which the test system was extracted and by either the natural vegetation in the ecosystem or the selection of crops for planting. Vegetation and crop selection are constrained and determined by the size (width and depth) of the soil core extracted.

Boundaries - The boundaries of the test system are determined by the size of the core and the growth space needed for the vegetative component.

Light - Light for the test system can be supplied by artificial lighting in either a growth chamber or greenhouse or can be the natural photoperiod occurring in a greenhouse. Because of the flexability of this test guide, lighting period is not specified; however, if the test is performed in a growth chamber, the daily photoperiod should be at least the average monthly incident radiation (quantity and duration) for the month in which the test is being performed. During extremely short

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natural photoperiods, which might not allow for l=flowering or seed set to occur, the photoperiod should be artificially lengthened. If the test is performed in a growth chamber, the daily photoperiod should be at least the average monthly incident radiation (quantity and duration) for the month in which the test is being performed.

Water - Water for the test system should either be purified untreated laboratory water with a known chemical composition or should be precollected, filtered rainwater from the site being evaluated. ASTM D19 Test Methods for Water Quality Analysis should be followed.

Soil - The soil for the microcosm should be an intact, undisturbed (non-homogenized) core extracted from a soil type typical of the region or site of interest and be of sufficient depth to allow a full growing season for the natural vegetation or the crops selected, without causing the plants to become significantly rootbound. Disturbances during extraction and preparation should be kept to a minimum. It should be noted that soil characteristics will play an important role in how the microcosm responds to a test substance. In addition, within-site soil heterogeneity will also influence the microcosm response. The approach used in this test system however, is based on comparison of responses among and between treatments rather than on the absolute values measured for an individual microcosm.

Biota - The biota of the microcosm are characterized by the organisms in the soil at the time of extraction (Parkinson et al. 1971; Phillipson 1971) and by the natural vegetation or crops introduced as the autotrophic component. The biota includes all heterotrophic and carnivorous invertebrates in the soil and all soil and plant bacteria, fungi, and viruses.

4.0 SIGNIFICANCE AND USES

4.1 This test method establishes a procedure to assist industry in evaluating the potential ecological impacts and environmental transport

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of a chemical that may be released or spilled in the environment. The suggested test procedures are designed to supply site specific information for a chemical without having to perform field test.

4.2 Specifically, this test is used to determine the effect of a chemical on; (1) growth and reproduction of either natural vegetation or crops, and (2) nutrient uptake and cycling within the soil/plant system. Additionally, the soil-core microcosm will provide information on; (1) potential for bioaccumulation (i.e., enrichment) of the chemical into plant issues, and (2) the potential for and rate of transport of the chemical through soil to ground water.

4.3 The results of this test should be used in conjunction with information on the chemical and biological activity of the chemical of interest to compare the relative environmental hazard and the potential for environmental movement once released.

4.4 The test methods described here are designed specifically for liquid or solid type materials. Significant modifications of the exposure system would be necessary to accommodate chemicals that may be released in a gaseous or aerosol form.

4.5 Correlation of multi-year soil-core microcosm test results with data derived from a series of multi-year field plot tests has been determined for a limited number of materials. Information relating to microcosm to field correlation can be found in Van Voris et al. (1984), Van Voris et al. (1985) and Tolle et al. (1983)

5.0 CHEMICAL CHARACTERIZATION OF TEST SUBSTANCE AND SOIL

5.1.1 *Information Required on Test Substance* - Minimum information required to properly design and conduct an experiment on a test chemical includes the chemical's source, composition, degree of purity, nature and quantity of any impurities present, and certain physiochemical information such as water solubility and vapor pressure at

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25°C (Laskowski et al. 1982; Swann et al. 1983). Ideally, the structure of the test chemical should also be known, including functional groups, nature and position of substituting groups, and degree of saturation. Octanol-water partition coefficient, dissociation constant, degree of polarity, and pH of both pure and a series of serial dilutions shall also be known. Where mixtures are involved or where a significant impurity (>1%) occurs, data must be available on as many components as practicable. However, the octanol-water partition coefficient (Kow or P) stands out as the key value. In combination with other chemical characteristics, log can be used to estimate Henry's Law Constant to predict volatility from soil solutions. Soil sorption can be estimated from log, the organic matter content, or from those data that suggest a relationship exists. Water solubility can be predicted with some degree of accuracy from log if this value is less than 7.

5.1.2 Several tests may be needed to supply information on environmental mobility and stability. Support information on phytotoxicity, the physicochemical nature of the chemical, its mammalian toxicity, invertebrate toxicity or its ecological effects (e.g., species-specific LD_{50}, biodegradability) not only assist in proper design of the microcosm experiment, but also are useful in assessing the fate and effects of the chemical in a terrestrial microcosm. If the chemical is radioactively labeled, the position and specific element to be labeled should be specified.

5.1.3 It is imperative to have an estimate of the test substance's toxicity to mammals as a precaution for worker safety. In addition, hydrolysis or photolysis rate constants should be known in order to determine necessary handling precautions. When a radiolabeled material is used, normal laboratory techniques for radiation safety provide an ample margin of safety (Wang and Willis 1965), except for chemicals in the "very highly toxic" category (i.e., rat oral LD_{50} <1 mg/kg).

5.1.4 The above information required for a test chemical is considered to be the minimum that is necessary to handle and apply the

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chemical safely (Walters 1980), as well as provide a perspective from which to interpret the test results. Water solubility, soil sorption and octanol-water partitioning, and vapor pressure largely will control the physical transport and bioavailability of a test chemical in soil. Water soluble chemicals are likely to move with soil water into the water films surrounding soil particles and root surfaces. Most microbially-mediated biodegradation occurs in the water-containing microsites of soil particles. Plant uptake and bioaccumulation is largely a function of water transfer to roots and solubility in fatty tissues. In addition, water-soluble chemicals and their transformation products may be leached to ground water. Water solubility of an organic chemical is a function of the dissociation of ionic compounds and the polarity of non-ionic compounds.

5.2.1 *Information Required on Soil* - Soil sorption of an organic molecule depends on several properties of the chemical (i.e., molecular size, ionic speciation, acid-base properties, polarity, and nature of functional groups) and of the soil (e.g., organic matter content, clay content, clay mineralogy and nature, pH, water content, bulk density). Highly sorbed chemicals may displace inorganic nutrient ions from exchange sites in the soil and also may be effectively immobilized, depending on soil pH. Thus, chemicals attracted more strongly to soil surfaces than to water may be very immobile in soil. In some cases, this may render the compound relatively resistant to biodegradation. In other cases, however, immobilization of the compound on soil particles may render it susceptible to extracellular enzymatic degradation. Specific information on descriptive data required for soil can be found in Section 6.2.2.

5.2.2 Compounds with very high vapor pressures (boiling point <80°C or vapor pressure >25 mm Hg) are not suitable for testing in the terrestrial soil core microcosm as described here. Modification of the test system such as that described in Van Voris et al. (1977) and Van Voris et al. (1981) should be useful for handling gaseous or aerosol type chemicals.

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6.0 TERRESTRIAL MICROCOSM EXTRACTION AND MAINTENANCE

6.1 *Microcosm and Cart Design* - The 60-cm-deep by 17-cm-diameter terrestrial microcosm is designed to yield pertinent information about a chemical for either a natural grassland ecosystem (Figure 1) or an agricultural ecosystem planted with a multiple-species crop (Van Voris et al. 1982, 1984, 1985; Zwick et al. 1984). The agricultural microcosm is a 17-cm-diameter tube of Driscopipe® containing an intact subsoil core (40 cm) covered by homogenized topsoil (20 cm). Driscopipe® is an ultra-high molecular weight, high-density, non-plasticized polyethylene pipe.

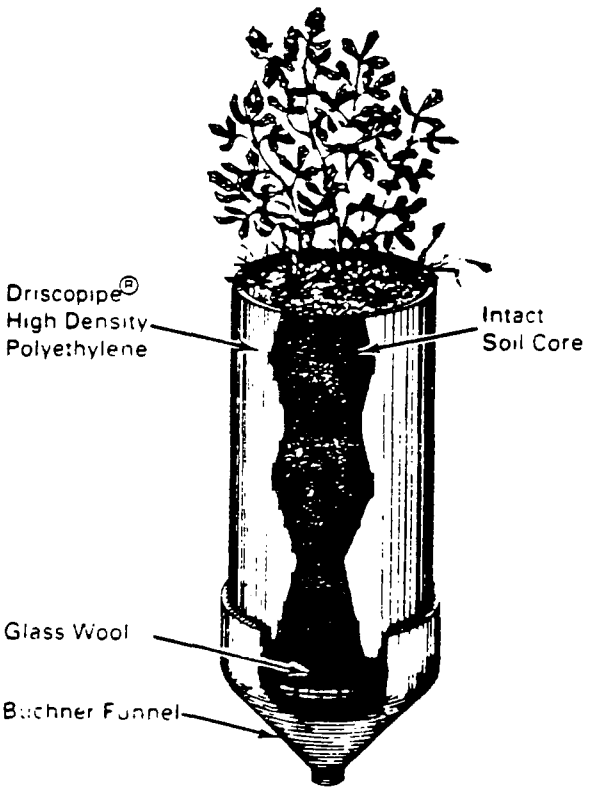

Figure 1. Microcosm Structure and Materials
(After Van Voris et al. 1985)

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non-plasticized polyethylene pipe. It is impermeable to water, lightweight, tough, rigid, and highly resistant to acids, bases, and biological degradation. Additionally, Driscopipe® does not release plasticizers or other compounds that may interface with test results. The tube sits on a Buchner funnel covered by a thin layer of glass wool. The funnel and tube are washed with 0.1 HCl prior to use and are reuseable for future tests.

6.1.2 Six microcosms are typically contained in a moveable cart, which is packed with Styrofoam® beads to reduce drastic changes in temperature profile (Van Voris et al. 1982, 1984;) (Figure 2). Cart dimensions are based on the environmental chamber size or the maneuverability required in the greenhouse. Carts are mounted on wheels so the cart can be moved within the greenhouse in a manner defined by statistical requirements to make exposure to light and temperature more uniform.

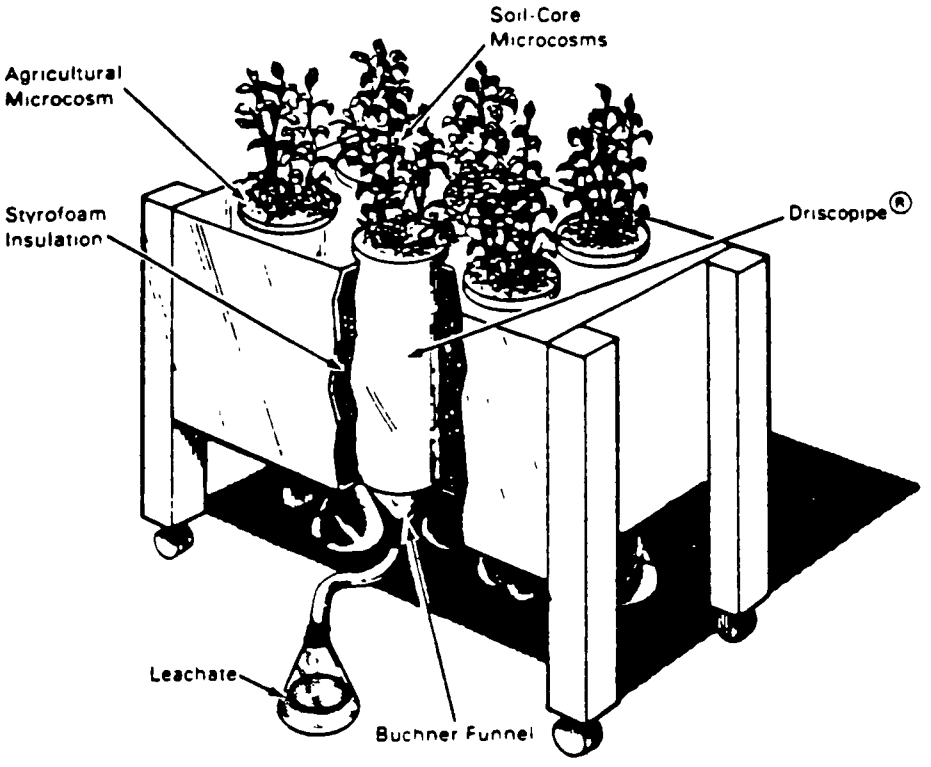

Figure 2. Arrangement of Microcosms in Styrofoam® Filled Cart (After EPA Test Protocol (In Press))

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6.2.1 *Soil Core Extraction* - Soil cores are extracted from either a natural grassland ecosystem or a typical agricultural soil in the region of interest. The intact system is extracted with a specially designed, steel extraction-tube (Van Voris et al. 1982, 1983, 1984; 1982; Zwick et al. 1984) (Figure 3) and a backhoe. The steel extraction- tube encases the polyethylene Driscopipe® to prevent the tube from warping and/or splitting under pressures created during extraction. For the agricultural microcosm, the plowed topsoil is moved aside and saved. Once the core is cut by the leading edge of the driving tube, it is forced up into the microcosm tube. For the natural grassland ecosystem, the vegetation is clipped before the core is extracted. The soil core microcosm is later removed as a single unit (soil and Driscopipe®) from the extraction tube and taken to the laboratory. For the agricultural microcosm, the topsoil

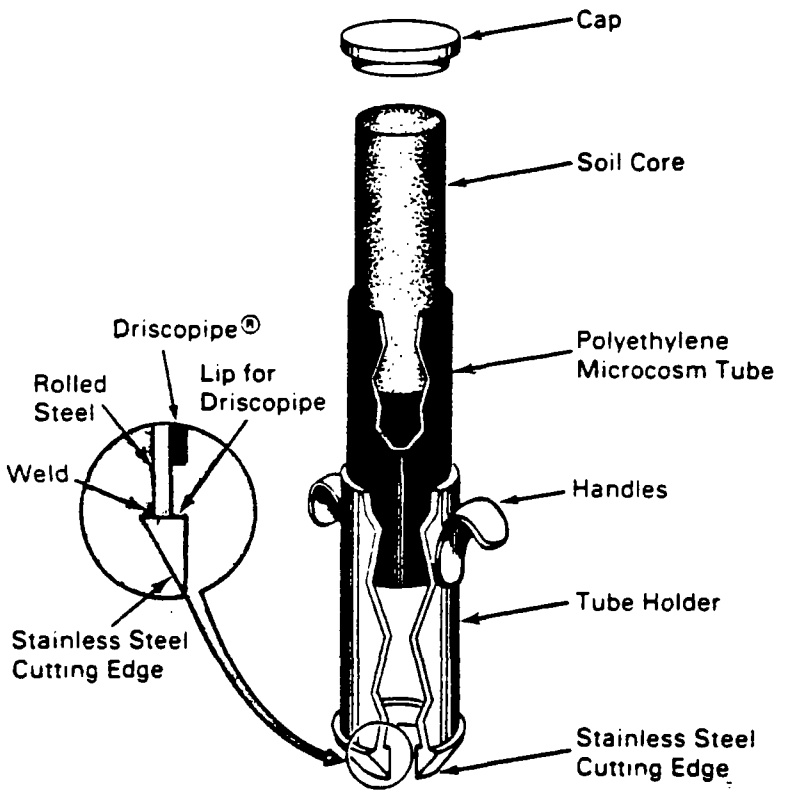

Figure 3. Diagram of Microcosm Extraction Tube (After Van Voris et al. 1985)

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is backfilled into the upper 20 cm of the microcosm tube. It is recognized that this extraction procedure does disrupt and compress the soil-core to a certain extent; however, the tests are being performed to compare different treatment levels and controls within the experiment.

6.2.2 Detailed chemical and physical properties of the soil in the test systems need to be determined using USDA description nomenclature. Information such as pedologic identity, according to the USDA 7th Approximation Soil Classification System, percent organic matter, field capacity, cation exchange capacity, bulk density, macro- and micro-nutrient content, organic matter content, mineralogy, exchange capacity, particle size distribution, hydraulic characteristics, and other important characteristics need to be measured before and after the experiment (Black 1965; 1982; ASTM D422-63, D2167-84, D2216-80, and D2488-84). The history of the soil, including previous crops grown and pest control and other management practices used, should be documented in order to assist in the interpretation of the results.

6.3.1 *Microcosm* - For the natural ecosystem (undisturbed grassland) test system, natural plant cover should be sufficiently diverse to be representative of plant species in the ecosystem of interest. When the agricultural microcosm is used, a mixture of grasses and broad leaves (e.g., legumes) should be included. Two or three species of grasses or legumes that are typically grown together as an agricultural crop in the region of interest should be chosen. The species chosen must be compatible regarding growth habit and be able to grow to maturity in the small surface area (240.5 cm^2) of the microcosm. In some cases, it may be appropriate to select a grain crop in order to evaluate the uptake of the radiolabeled test substances and their degradation products in grain normally grown for human consumption (Van Voris et al 1984).

6.3.2 The seed application rate should duplicate standard farming practice for the region of interest. Seeds should be planted evenly and covered with an appropriate depth of soil. Similarly, the test substance application form should approximate a reasonable scenario of how the test

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substance might arrive at the site in question. If the test substance is a solid, one option is to mix it with the topsoil prior to planting, thus mimicking the plowing of an agricultural field prior to sowing seed. Alternatively, it may be dusted on the surface to simulate dry deposition.

6.3.3 Plant tissue analysis should be consistent with those practices that may be region specific. Plants from either the natural grassland ecosystem or the agricultural test unit are harvested from each microcosm at the end of the test period (Van Voris et al. 1982; Tolle et al. 1982), and air dried, and then oven-dried. In the range-finding test (see Section 7.3.1), the crop is harvested four weeks after first exposure to the test substance. In the definitive test (see Section 7.4.1), plants may be harvested one or two times during the 12-week growing period or at the end of the test. The definitive test may need to be extended beyond the 12-week test period to accommodate plant species which take longer to reach the desired maturity (e.g., seed production).

6.4.1 *Microcosm Watering and Leachate Collection* - Microcosms are watered as dictated by a predetermined water regime based on site history with either purified laboratory water (e.g., distilled, Reverse Osmosis) or with rainwater that has been collected, filtered and stored in a cooler at 4°C (Van Voris et al. 1980, 1982, 1983). If comparisons are being made between microcosms and field plots, then parallel watering in both units should be attempted. Care needs to be taken to maintain sufficient water, but over-watering can induce fungal disease and stress.

6.4.2 Microcosms are leached at least once before and once every two or three weeks after dosing. However, if natural rainfall amounts are higher or lower this should be used to guide selection of a leaching regime. Caution should be exercised to not over water because this may drastically alter the rate of degradation, transformation, translocation and transport within the microcosm.

6.4.3 Leachate is collected in 0.1N HCl washed flasks after excess laboratory water or rainwater has been added to each microcosm. The 500-ml flask is attached to the Buchner funnel with Tygon tubing,

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(see Figure 2) in an attempt to insure that all test microcosms will leach within a 2-day period, 15% more microcosm soil cores are extracted than are required for a combination of both the range finding and definitive tests. When the microcosms are leached before planting, those which do not leach, leach too quickly, or take longer than two days to produce 100 ml of leachate after the soil has been brought to field capacity are discarded.

6.5 *Greenhouse and Growth Chamber Environments* - Microcosms in insulated carts or other such devices are kept in a greenhouse or environmental chamber that has temperature and light control. Greenhouse and environmental chamber temperatures are designed to approximate outdoor temperatures that occur during a typical growing season in the region of interest. If the experiment is not conducted in the greenhouse during the normal agricultural growing season, then lights suitable for plant growth on timing devices are used to simulate the photoperiod and intensity typical for the growing season in the area of interest. If the experiment is conducted in the greenhouse during periods when the photoperiod of the natural light is not long enough to induce flowering and seed set, then supplimental lighting will be required.

7.0 TEST PROCEDURES

7.1 *Test Purpose and Assumptions* - The purpose of the terrestrial soil-core microcosm test is to determine the fate and ecological effects of a test substance, including transformation products, within a site- specific natural grassland or agricultural ecosystem. The relationship of fate and effects data from treated versus control microcosms is assumed to be very similar to that from treated versus control field test plots (Van Voris et al. 1982, 1983, 1984). This assumption is supported by the microcosm/field comparisons reported by Van Voris et al. (1982, 1984), Jackson et al. (1979) and Tolle et al. (1983). The fate and effects from the microcosm test should then be related to

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either the natural or agricultural ecosystems which have the same combination of soil type, vegetation, crop species, and environmental variables used during the microcosm test.

7.2.1 *Evaluation of Test Substance* - Physicochemical information supplied for the test substance (see Section 6.1.1) is used to tailor the general range-finding test procedures to the specific substance. Phytotoxicity and/or bacteriostatic action, if known, should be taken into account in designing the exposure concentrations of the range-finding experiment. Only one concentration above that known to cause at least 50% change in plant growth or 50% change in bacterial growth/respiration needs to be tested. In addition, the lowest treatment level should not be lower than a factor of 10 in soil and 100 in water. These factors are the analytical limits of detectability of the parent compound at the start of the experiment.

7.2.2 The water solubility and soil sorption capacity can be used to determine the appropriate frequency of leachate analyses for the radiolabeled test substance and its transformation products. This same information will also determine the design of the soil sampling procedures for the range-finding test. Chemical structure and any degradation information are used to determine which transformation products to analyze for in the soil, leachate, and plant tissue collected.

7.2.3 As was stated in Section 6.3.2, exposure should approximate a reasonable scenario. The water solubility, dissociation constant(s), and soil pH must be taken into account in determining the formulation of the chemical. The maximum concentration of a chemical in water solution should not exceed half of saturation. However, solubility may be markedly altered by ionization in soil. If the soil pH is such that a more soluble form is likely, the test substance should be adjusted accordingly with either sodium hydroxide or hydrochloric acid prior to introduction into the microcosm. If the pH adjustment to increase solubility is extreme ($4<pH>9$), chemical and photolytic degradation may be enhanced when

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preparing the chemical solutions. In all cases, the exposure formulation should be consistent with the hypothetical scenario forming the basis for the test.

7.3.1 *Range-finding Test Experimental Design* - The range-finding test should last four weeks from first exposure of the test substance to plant harvest. At the start of the test, the microcosms are dosed (see Section 7.5) with a minimum of five concentrations of the test substance. Three replicate microcosms are used for each of the four or five treatment levels and the controls, resulting in a total of 15 or 18 microcosms. Concentrations typically used for dosing are 0.1, 1.0, 10, 100 ppm, if a hypothetical scenario is not known and, if deemed appropriate, 1000 g/g of topsoil in the upper 20 cm of the microcosm. The logarithmic scale for dose levels in a range-finding test is suggested by Rand (1980). The bulk density (g/cm^3) of the dry topsoil is used to calculate the exposures. Depending on whether the potential mode of release of the test chemical is likely to be a single accidental spill or a repeated effluent release. Either a single, "acute" dose, or a multiple application, "chronic" dose, should be considered and selected based on a reasonable exposure scenario. In either case, the total amount of test chemical applied should be equal to five of the recommended test concentrations.

7.3.2 Each microcosm cart, holding one replicate of each of the four or five test concentrations and a control, is moved randomly on a weekly basis in the greenhouse to avoid location induced effects. If no discernible greenhouse position effects are recorded during the range-finding test, then this process need not be followed during the definitive test.

7.3.3 The range finding tests yield two necessary types of information. These include: (1) estimates of the bounds of toxicity within which the 50% response (e.g. LD_{50}) lies and (2) initial estimates of variance in response. Given the identification of bounds of toxicity for the initial doses, the dose levels for the definitive tests may be refined. The variance estimates should be used to determine sample sizes

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needed in the definitive tests to achieve statistical tests able to detect specified differences (Δ) among dose levels with a specified power (1-B).

7.4.1 *Definitive Test Experimental Design*- The definitive test lasts for 12 or more weeks from first exposure of the test chemical to final harvest. Test results may be influenced by extraneous environmental sources of variation, such as temperature or light gradients within a greenhouse. These sources of variation may be accounted for by randomly repositioning the cards (see 7.3.2) and/or by employing experimental designs such as randomized block, latin-square, or other more complex designs. If such extraneous sources of variability in test results are not accounted for, results may be biased, thus jeopardizing the outcome of the experiment. The types of statistical analyses to be performed are decided at this point, dictated largely by the experimental and treatment designs. The experimental designs determine the method of randomization of the treatments to account for extraneous sources of variability in the experiment environments, while the treatment design determines number of treatments and the arrangement of treatments with respect to one another.

7.4.2 At the start of the test, the microcosms are dosed with three concentrations of the test substance. The number of microcosms to be dosed should be determined by the desired power of the statistical tests. Power is influenced by the variance of the response (estimated from range finding tests), the size of the difference to be detected among the treatments, and the alpha (x) level. The desired power, alpha level and detectable difference are specified by the researcher, the variance estimates are obtained from the range finding tests. Based on these four values, the sample size, or number of replicates for each dose level should be determined (see Neter and Wasserman, 1974 and Kasterbaum avel Hoel, 1970 for discussion of power of a test). The three treatment levels chosen are estimated from the range-finding test data to produce a 20 to 25% change in productivity for each subsequent concentration of the test chemical. In order to reduce analytical costs associated with the fate studies, the replicate microcosms in each treatment level are employed as

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replicate pairs. Thus, leachate and plant tissue analyses are conducted on the polled samples from paired microcosms. Productivity data, on the other hand, are analyzed for each individual microcosm. Each cart holds six to eight microcosms (see Figure 2). The microcosms paired for analyses are placed in different carts to insure that all microcosms are housed under the most uniform conditions possible.

7.4.3 *Plant Productivity* - Depending on the type of natural vegetation or crop planted, it may be possible to harvest more than once (e.g., during the middle and at the end of the test). If growth is vigorous, grasses may be harvested at a pre-arranged height, e.g., 2 to 6 cm above soil surface during the middle of the 12 week test period. Multiple harvests permit evaluation of both gross plant yield and plant uptake of the test substance with respect to time (Elseewi et al. 1980; Van Voris et al. 1984, 1985).

7.5.1 *Exposure Techniques* - If the primary mode of exposure of the test chemical is anticipated to be by addition of pH adjusted laboratory water or rainwater containing appropriate concentrations of the test substance, then this procedure should be followed. Again, the following procedure is used unless information is available that suggests some other regime should be used or that the chemical is associated with other chemicals or solvents. In no case shall the total aqueous volume of exposure be sufficient to cause microcosm leaching (i.e., > field capacity). Recommended concentration levels are discussed in Sections 7.3 and 7.4. Test substances which are likely to be released into the environment as a liquid or powder, and which can be mixed with water, are applied as a single dose of liquid sufficient in volume to bring the microcosm soil surface horizon to field capacity. The volume of laboratory water or rainwater required for exposure can be determined on an unplanted microcosm of the same soil type on site; the volume selected should be identical for all microcosms. Carriers other than water are not recommended unless they are likely to be released into the environment in conjunction with the effluent stream or accidental spill of the test

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substance. If a carrier is necessary, then acetone or ethanol should be considered; however, the use of carriers should be avoided unless they are essential to produce a realistic exposure.

7.5.2 Several typical exposure modes are suggested for particular types of test substances if either a hypothetical or real (actual) exposure scenario is not available. If the test substance is likely to be a contaminant of irrigation water, it should be applied every week in proportionate concentrations, such that the total amount applied equals the desired treatment level. If the test substance does not mix with water, it should be applied as evenly as possible to the top of the unplanted microcosm and mixed into the topsoil prior to planting. If the test substance is normally sprayed on growing plants (e.g., pesticide), then the desired amount should be mixed with the volume of water necessary to wet the soil surface and wet the plants to the point where they begin to drip. A chromatography sprayer or nebulizer should be used to spray plants that are past the seedling stage. The recommendations by the test substance's manufacturer for field spraying should be followed as closely as possible, but the test should be terminated (last harvest) at least eight weeks after the plants are sprayed.

7.6.1 *Waste Disposal* - All liquid (leachate) and solid (soils and plant tissues) samples must be retained for proper disposal. All sample collection bottles, collection apparatus, microcosm tubes (Driscopipe) and sampling tools should be thoroughly cleaned (acid washed) and analyzed for radioactive contamination before they are stored or used on another test system. All samples and the remaining, undisturbed portion of the test system should be disposed of in accordance with EPA and Nuclear Regulatory Commission (NRC) regulations, if radiolabeled compounds were used. Soil leachate and all other aqueous-sample wastes should be treated prior to disposal using one or more of the following techniques:

(a) filtration

(b) activated charcoal filtration

(c) ion exchange.

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7.6.2 Soils contaminated with organic residues and/or radiolabeled compounds as well as the Driscopipe , sample bottles, glassware, gloves, masks, filters, activated charcoal from aqueous cleanup, and any other potentially contaminated equipment must be either certified as uncontaminated or packaged and disposed of in accordance with existing EPA and NRC guidelines and regulations.

8.0 FATE AND EFFECTS SAMPLING PROCEDURES

Sampling procedures have been divided into two basic categories: ecological effects sampling and test-chemical fate sampling. Ecological effects sampling may include productivity measurements, physical appearance of plants, and nutrient loss or uptake measurements. Test-chemical fate sampling may include leachate, soil, and plant analyses.

8.1.1.1 *Ecological Effects Sampling - Productivity Measurements* - Primary productivity is a commonly measured parameter in terrestrial effects testing. Depending on the plant species, it may be desirable to report total yield or yield by plant part. For example, in the case of grain crops such as soybeans, oats, and wheat, the total biomass yield can be reported in addition to the grain yield. This will allow a relative comparison of total biomass yield with grain yields typically reported for local agriculture. In addition, separate grain samples may be useful for later tissue analyses to determine whether the test chemical was enriched in potentially edible plant parts. For other systems, such as natural grassland microcosms, segregation into plant parts may be unnecessary.

8.1.1.2 At a minimum, productivity should be reported as oven-dry weight. Jones and Steyn (1973) recommend 65°C for 24 hours as adequate conditions for drying without unnecessary thermal decomposition of plant material. Information on the chemical's volatility should be evaluated when selecting a drying temperature. It may be desirable in some circumstances to report air-dry productivity or to be able to calculate

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air-dry yields based on moisture loss after oven-drying. These data could be useful if agricultural crops are the plants used in the microcosm and if it is desirable to compare productivity with yields reported in local agriculture.

8.1.1.3 The number of harvests will depend on the plants grown. An agricultural crop, alfalfa/timothy for example, may require two or more harvests over the course of the testing period (Malanchuk et al. 1980; Van Voris et al. 1984).

8.1.2 *Physical Appearance of Plants* - Throughout the test period, it is desirable to record the physical appearance of plants in the terrestrial microcosm. Symptoms of nutrient deficiency or toxicity, pathogenicity, water stress, or test-chemical-induced toxicity should be monitored. These observations may be useful in interpreting the specific ecological effects of a test chemical relative to responses in plants elicited by known environmental toxicants or stresses (Daubenmire 1959). Careful observation on physical appearance in controls vs. treated microcosms may also aid in determining whether abnormal physical appearance is a result of the test chemical or is a manifestation of microcosm management.

8.1.3.1 *Nutrient Loss Measurements* - An important ecological effects sampling procedure is to monitor nutrient losses in leachates (O'Neill et al. 1977; Van Voris et al. 1980; Jackson et al. 1978; Jackson et al. 1979). The rationale for such monitoring is explained in detail in the accompanying support document. One of the desirable attributes of the terrestrial microcosm approach to testing chemicals is the relative ease with which soil leachates can be collected. This offers the potential to construct nutrient budgets for the model ecosystem (Schindler et al. 1980; Gile and Gillett 1979).

8.1.3.2 The final suite of nutrients monitored in leachates probably will depend on the nature of the test chemical Ausmus et al. 1978; Ausmus et al. 1979; Jackson et al. 1979; Harris et al. 1980). Those nutrients which initially should be considered during the range-finding

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test include calcium, potassium, nitrate-nitrogen, ortho-phosphate, ammonium- nitrogen, and dissolved organic carbon (DOC). Depending on the results of nutrient losses measured during the range-finding test, a set of nutrients can be selected for monitoring during the definitive test.

8.1.3.3 Various methods exist to analyze for the nutrients. Standard techniques which have proven useful include atomic absorption spectrophotometry for Ca and K and analysis using a Technicon Autoanalyzer II for nitrate-nitrogen, ortho-phosphate, DOC, and ammonium-nitrogen (ASTM D511-84, D515-82, D1254-67, D1426-79, and D3867-79; Association of Official Analytical Chemists (AOAC) 1975; American Society for Testing and Materials 1979; EPA 1979). For less rigorous determinations, e.g., during the range-finding test, ion-specific electrodes may be useful for nitrate- and ammonium-nitrogen.

8.1.3.4 A standard procedure, described below, has proven to be useful in handling leachates. As soon as soil water samples are collected, the sample volume is recorded and the pH determined using a glass electrode. Samples are centrifuged at low speed (e.g. 5000 rpm) to remove large particulates and filtered through a 0.45-micron filter. The sample should be divided into two aliquots prior to storage in the dark at 4°C. Blanks consisting of distilled water and reference standards in instrument calibration quantities should be prepared and stored similarly.

8.2.1 *Test-Chemical Fate Sampling* - The fate of the test chemical (see EPA 1982b) will be determined by methods appropriate to the test, including sensitivity factors adequate to verify exposure and distinguish between parent material, transformation products, and naturally occurring materials present in the test system. Usually this will involve use of a radiolabeled parent compound and subsequent analysis of microcosm components for radioactivity and chemical identity. Methods appropriate to the latter may be adequate for quantification of fate, but usually cannot reveal bound residues in soil or plants and frequently are inadequate for cost-effectively tracing movement and transformation. To the extent that the fate in soil and plants is well enough understood from other experiments and depending on the degree to which the microcosm test

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is being employed to verify fate and exposure hypotheses, analytical requirements may be reduced (Harvey 1983; Lichtenstein et al. 1972; Lichtenstein et al. 1973; Metcalf et al. 1973; Cole et al. 1976).

8.2.2 *Radiolabeling the Parent Compound* - The parent compound may be labeled with ^{14}C either in an appropriate aromatic, cyclic carbon group, or linear chain (Lichtenstein et al. 1974). Other labels, including stable isotopes such as ^{15}N may be more useful and informative. In order for the microcosm test to permit an analysis of the fate of the parent compound and/or its metabolites, attention should be paid to known or hypothesized metabolic pathways for test substances. Hence the site and form of label is an integral part of the total test design. The laboratory conducting the test is not required to have the capability for radiolabeling, since this is routinely handled by speciality chemical firms. Sufficient radioactivity must be present in order to detect at least 1% of the initial parent compound in a typical sample of leachate, soil or plant tissue.

8.2.3.1 *Compartment Analysis for Labeled Compounds* Several compartments of the terrestrial microcosm can be analyzed for radioactivity, which include: samples of soil leachate, plant tissue, including roots and shoots; and soil from different depths. The different soil depths used for radiochemical analyses should be selected based on information on soil sorption of the compound of interest. From previous experience, these depths should be relatively close to the soil surface (1-2 cm) for radiolabeled chemicals that are strongly sorbed to soils; if any isotope appears in the leachate, the depth selection should be lower in the soil profile. Samples will be homogenized and extracted with solvents appropriate for the parent compound. Additional extraction steps may be necessary such as acidification and extraction with non-polar solvents, sohxlet extractions with polar and/or non-polar solvents, alkaline or acid hydrolysis with or without heat, detergent extractions, or protease digestion. The ^{14}C in the soil or plant samples which cannot be extracted will be oxidized and analyzed as $^{14}CO_2$ as described by

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Lichtenstein et al. (1972) or dissolved in Protosol and Aquasol as described by Cole et al. (1976). The extracts and the oxidized or dissolved samples will be counted by ^{14}C liquid scintillation (Metcalf et al. 1973; Cole et al. 1976).

8.2.3.2 At the termination of the range-finding test, soil samples will be collected from the top, middle, and bottom of the 60-cm soil cores. If the labeled compounds or its metabolites are not detected by liquid scintillation in the deeper soil samples, then soil samples at the end of the definitive test should be taken closer to the top of the soil column.

8.2.4 *Identification of Degradation Products* Liquid scintillation will identify the presence of ^{14}C-labeled compounds in sample extracts, but the identification and quantification of the parent compound or its degradation products will require gas-liquid chromatography (GLC), and thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC) (U. S. EPA 1982b). TLC autoradiography using no-screen X-ray film for chromatographed fractions found to be radioactive by liquid scintillation counting (Cole et al. 1976; Metcalf et al. 1973) is most cost-effective. Whenever possible, the identity of the parent compound and probable degradation products in fractions found to be radioactive by liquid scintillation counting (Cole et al. 1976; Metcalf et al. 1973) will be verified by gas-liquid chromatography methods. Also, the concentration of the parent compound and degradation products should be verified by an alternative chromatographic methods system (e.g., HPLC or GLC) with known standards.

9.0 DATA ANALYSIS

9.1 *Ecological Effects Analysis* A combination of statistical analysis procedures are suggested to evaluate the data collected to discern ecological effects. Regression, correlation, and covariance analysis, as well as analysis of variance (ANOVA) procedures described in

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the following section may be as appropriate methods for data analysis. Use of these or other analysis techniques will be determined by the objectives of the experiments and the original experimental and treatment designs. A number of statistical references describe the common methods of statistical analysis (Sokal and Rohlf 1981; Snedecor and Cochran 1980; Steel and Torrie 1980). The level of significance for all statistical tests of hypothesis is set at the 5% level (α = 0.05) and power of the test (1-B) at 0.90 or .095 unless otherwise defined. The results of all statistical tests performed on all data must be fully documented for evaluation. In addition, graphs displaying the data and tables containing all raw numbers should be appended to the submittal.

9.1.1 *Test Material Effects* - Because the mode of exposing the test system can be done without the use of carrier chemicals (see Section 6.2.3 and 6.5.1), there is no need to perform separate analyses on carrier effects as suggested in the marine and freshwater microcosm protocols. This may be mandatory however, if it becomes advisable to use carrier chemicals.

9.1.2 *Productivity of Natural or Planted Vegetation* The sum total of both air-dry and oven-dry biomass expressed in g/m² collected during and at the end of the definitive test should be evaluated initially by using side-by-side histograms displaying the calculated means, and 95% confidence intervals for controls and all exposure levels. This will allow early visual evaluation of the effects of the chemical by exposure level. Variance estimates may indicate whether logarithmic or some other transformation of the data may be necessary for graphic display and analysis. Analysis of variance (ANOVA) calculations (Sokal and Rohlf 1981; or Steel and Torrie 1980) should be carried out first to test for position effects within the carts and within the environmental area where the test was performed. Position effects may be accounted for in the design of the experiment (e.g., by blocking) and any effect of position can then be accounted for in subsequent analyses. If these tests prove to be significant at the 5% level (α = 0.05), then this will need to be

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accounted for in the remainder of the statistical analyses. Pair-wise comparisons of variables that are measured only once during the 12-week experiment may be necessary (Snedecor and Cochran 1980).

9.1.3.1 *Statistical Methods* - In order for any statistical procedures to be applicable, all experimental microcosms must be assigned to the experimental treatment level by some random process. This may be accomplished through use of a completely randomized experimental design, randomized block, Latin-square or other appropriate experimental designs. As stated earlier, if an appropriate experimental design is not used, the results and analysis may be biased, thus jeopardizing the outcome of the experiment. Analysis of variance (ANOVA) procedures should be performed on biomass data in order to determine if an ecological effect resulted from the treatment levels of the parent compound and transformation products. If the ANOVA is significant, then Orthogonal comparisons or a multiple-range comparison such as Duncan's should be performed in order to determine which of the treatment means were different from the others. The undosed controls are considered to be one of the treatment levels. Again, the 5% level (α = 0.05) should be considered as the level of significance for all tests and the power should be 0.90 or 0.95. All values, whether significant or not, must be reported for each statistical test being performed. Where more than a single factor or treatment is incorporated into the original experimental design a factorial ANOVA is the appropriate test to conduct.

9.1.3.2 Regression analysis should subsequently be performed on the productivity results. Outlier data, defined as an obvious data recording or reporting error, should be excluded; however, these data and the fact that they have been excluded must be reported. If a substantial number of data points have been declared as outliers, deficiencies in quality control may necessitate rerunning the test. Once outlying values have been detected and removed from further statistical evaluations, regression models or Probit analysis can be used to estimate the concentration where 50% of the productivity realized in the controls occurred for the test

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substance (EC_{50}). Ordinary linear least squares regression analysis may be performed to define the response of relevant production parameters as functions of dose. If it appears that productivity is nonlinear with respect to dose, it may be necessary to transform the data or fit either a quadratic or cubic least-squares regression model to the data for this type of response. Utilization of computer software packages such as SAS (Statistical Analysis System) or BMDP (Biomedical Computer Program) may prove useful.

9.1.4 *Physical Appearance of Plants* - Changes in physical appearance in plants in the terrestrial microcosms should be reported for all test units. No statistical evaluations of the effects of the chemical and transformation products on appearance are to be performed unless there is a clearly identifiable pattern of effects. In this case, clearly recognizable patterns of injury to plants may be ranked in terms of severity. A non-parametric test, such as Kruskal-Wallis test, may be conducted to test for differences in plant injury.

9.1.5.1 *Nutrient Losses* - Based on the nutrient or nutrients selected for analysis in the soil leachate (see Section 8.3.1) collected from each microcosm, the total cumulative nutrient loss for each test system for each nutrient should be calculated by (a) multiplying nutrient loss concentration from each collection date times the total volume leached from that microcosm for that collection date, and (b) then adding it to the previous sum of total loss. Plots of the collection date means (±SE) of the cumulative nutrient losses for each treatment level should be developed as a function of days after seeding for the agricultural microcosm or days after exposure for the natural grassland microcosm. Zero loss should be the starting point; if there was no leachate for any microcosm during a particular collection period, the data point should be recorded as zero so that no data are considered missing.

9.1.5.2 A one-way ANOVA should be performed on the total cumulative nutrient loss data at the end of the experiment, to evaluate the treatment level effects. A multiple comparison procedure, such as Duncan's, may be

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used to determine which specific treatment means were different from each other.

9.1.5.3 Regression/correlation analysis comparing nutrient losses versus productivity should be performed to determine the relationship between these two independently measured variables. In order to perform this analysis the productivity and cumulative leachate loss measures, must be matched unit for unit. This test should be performed for each nutrient analyzed versus productivity.

9.2 *Chemical Fate Analysis* - At the end of the experiment, the budget or distribution of the parent compound and transformation products is calculated for each exposure (concentration) level. This entails determining the amount added and the subsequent distribution of the radioactivity of the parent compound and transformation products through chemical analysis in each of the primary compartments, soil, H_2O, plant tissue and air of the test system as well as in the soil leachate. Fate analysis should result in distribution values for above-ground plant tissues, plant roots, each soil depth (see Section 8.2.3), and loss to soil leachate. Gaseous loss can be estimated. This is followed by performing statistical analyses for each exposure level on any differences in distribution of the compound throughout the test system. Multi-compartmental modeling and multivariable analyses such as multivariate analysis of variance may also prove useful in assessing the fate of parent compounds and transformation products.

9.2.1.1 *Radioactivity Budgets* - The calculation of a complete budget of all radioactivity is required to be submitted with the results of this test. The budget must show the percent of the compound that was tagged and the location of the tag. The label may be ^{14}C, stable ^{15}N, or another suitable label and should be located in a portion of the molecule expected to persist and/or have biological activity.

9.2.1.2 *Total Radioactivity Added* - The total radioactivity added per test unit is based on the decay rate of the radioactive tag, the total amount of radioactive tag added to the compound when initially formulated,

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the length of time between formulation and test unit exposure (radioactive decay), and the particular exposure level of the test unit.

9.2.1.3 *Total Radioactivity Removed* - The total radioactivity removed from the microcosm is based on the concentration of the radiolabel in (a) the soil leachate concentration times the volume of soil leachate lost per collection date, (b) the calculated gas phase losses of the compound, and (c) the type of radiolabel and the rate of radioactive decay of that label over the length of the experiment.

9.2.1.4 *Total Radioactivity Remaining* - The total radioactivity remaining in the microcosm is based on radiolabel analysis of each of the primary compartments (a) above-ground plant tissues, (b) plant roots (i.e., cleaned of soil particles) and (c) the distribution of the label through the different soil depths. The soil depths to be analyzed will be based on a range-finding evaluation as suggested in section 8.2.3.

9.3.1 *Fate of Parent Compound and Transformation Products* - Calculations should be made for each exposure (concentration) level relative to the percent distribution of the test substance for (a) above-ground plant tissues, (b) below-ground plant tissues, (c) each depth through the soil profile, (d) losses to soil leachate, and (e) calculated total vaporization or gaseous losses. In addition, an analysis of the time to reach steady-state loss of the chemical compound in the soil leachate and the time to initiate compound leaching from each exposure level should be calculated. For those exposure levels where the time to reach steady state loss in the soil leachate is greater than the length of the experimental period (12 weeks) then an extrapolation of a regression model may be used to estimate the time necessary to reach steady state (Snedecor and Cochran 1980). This should be done cautiously and with the assumption that the shape of the response beyond the experimental period is the same as that estimated for the experimental period.

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9.3.2 The calculations for the final distribution of the test material and transformation products are based on the measured radioactivity in that compartment on a per gram basis times the total weight or volume of material in that compartment, expressed on a dry weight basis where appropriate. All calculations, are subsequently corrected for the radioactive decay occurring since the beginning of the experiment. The quantities of compound are to be expressed as a percent of the original compound added to the test system. An ANOVA test should be performed on the calculated mean percentage remaining for each of the four main compartments to discern any differences among exposure concentrations. It may be necessary to transform the percentage data prior to ANOVA in order to satisfy the assumptions of the analysis. In addition, regression analyses should be performed comparing above-ground productivity versus concentration of parent compound and transformation products in above-ground plant tissues, and cumulative nutrient losses for each exposure concentration versus parent compound and transformation products in the soil leachate.

9.4.1 *Bioconcentration and Enrichment* - Calculate the concentration of the radioactivity in the above-ground plant tissues and in the top 15 cm of soil on a concentration per unit dry weight basis. The ratio of the plant tissue concentration to soil concentration is then defined as the Bioconcentration Factor (BF). Side-by-side histograms displaying the BF ratios should then be compared for statistical differences.

9.4.2 Enrichment ratios (ER) should be calculated for the higher exposure levels by dividing the activity expressed on a dry weight basis in a higher exposure level by the lowest exposure level in which a significant (i.e., detectable) concentration could be analytically determined. The enrichment ratio is then regressed against exposure concentration to determine the linearity of the uptake of the chemical as measured through the radiolabeled portion of the chemical. If it appears that an ordinary least squares regression analysis is not the appropriate function defining the enrichment of the chemical then an attempt to fit a quadratic or cubic equation should be made. The above guidelines for

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calculating BFs and ERs assume that no wet analytical chemistry has been performed to separate the parent compounds from the transformation products in each compartment. If this has been done, then individual BFs and ERs should be calculated as outlined above.

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10.0 TEST PROTOCOL BIBLIOGRAPHY

Association of Official Analytical Chemists. 1975. Official Methods of Analysis of the Association of Official Analytical Chemists. 12th Edition. Washington, D.C., 1094 pp.

American Society for Testing and Materials. 1979. Annual book of ASTM Standards. Part 31, Water. American Society for Testing and Materials, Philadelphia, PA. 128 pp.

Ausmus BS, Dodson, GF, & Jackson DR. 1978. Behavior of heavy metals in forest microcosms: III. Effects on litter-soil carbon metabolism. Water Air Soil Pollut. 10:19-29.

Ausmus BS, Kimbrough S, Jackson DR, & Lindberg S. 1979. The behavior of hexachlorobenzene in pine forest microcosms: transport and effects on soil processes. Environ. Pollut. 20:103-111.

Black, CA (ed). 1965. Methods of Soil Analysis I. Physical and Minerological Properties. American Society of Agronomy, Madison, WI. 770 pp.

Black, CA (ed). 1982. Methods of Soil Analysis II. Chemical and Microbiological Properties. 2nd Edition American Society of Agronomy, Madison, WI. 802 pp.

Cairns J, Jr. 1981. Testing for effects of chemicals on ecosystems. National Academy of Science, Washington, D.C. 103 pp.

Cairns J, Jr. ed. 1985. Multispecies Toxicity Testing. Pergamon Press, New York. 253 pp.

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Cole LK, Sanborn JR, & Metcalf RL. 1976. Inhibition of corn growth by aldrin and the insecticide's fate in the soil, air, crop, and wildlife of a terrestrial model ecosystem. Environ. Entomol. 5:583-589.

Daubenmire, R. 1959. A canopy cover method of vegetation analysis. N.W. Science 33: 43-64.

Elseewi AA, Straughan IR, & Page AL. 1980. Sequential cropping of fly ash-amended soils: Effects on soil chemical properties and yield and elemental composition of plants. Sci. Tot. Environ. 15:247-259.

Environmental Protection Agency. 1979. Manual of Analytical Methods for the Analysis of Pesticide Residues in Human and Environmental Samples. Health Effects Research Laboratory. Research Triangle Park, N.C.

Environmental Protection Agency. 1982a. Environmental Effects Test Guidelines. Washington, DC. Office of Pesticides and Toxic Substances, U.S. EPA. EPA 560/6-82-002. Available from NTIS, Springfield, VA PB82-232992.

Environmental Protection Agency. 1982b. Chemical Fate Test Guidelines. Washington, DC. Office of Pesticides and Toxic Substances, U.S. EPA. EPA 560/6-82-003. Available from NTIS, Springfield, VA PB82-233008.

Environmental Protection Agency (In Press). Experimental Terrestrial Soil-Core Microcosm Test Protocol. A Method for Measuring the Potential Ecological effects, Fate, and Transport of Chemicals in Terrestrial Ecosystems. Corvallis Environmental Research Laboratory Research Report 71.

Gile JD, & Gillett JW. 1979. Fate of selected fungicides in a terrestrial laboratory ecosystem. J. Agric. Food Chem. 27:1159-1164.

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Harris WF, Ausmus BS, Eddlemon GK, Giddings JM, Jackson DR, Luxmore RJ, O'Neill EG, O'Neill RV, Ross-Todd M, and Van Voris P. 1980. Microcosms as potential screening tools for evaluating transport and effects of toxic substances. ORNL/EPA-4, EPA-600/3-80-042. Environ. Sci. Div. Publ. No. 1506, Oak Ridge, TN. 453 pp.

Harvey J, Jr. 1983. A simple method of evaluating soil breakdown of ^{14}C pesticides under field conditions. Residue Rev. 85:149-158.

Jackson DR, Selvidge WJ, & Ausmus BS. 1978. Behavior of heavy metals in forest microcosms: II. Effects on nutrient cycling processes. Water Air Soil Pollut. 10:13-18.

Jackson DR, Ausmus BS, & Levin M. 1979. Effects of arsenic on nutrient dynamics of grassland microcosms and field plots. Water Air Soil Pollut. 11:3-12.

Jones JB, & Steyn WJA. 1973. Sampling, handling, and analyzing plant tissue samples, pp. 249-270. In: LM Walsh and JD Beaton eds. Soil Testing and Plant Analysis. Soil Science Society of America, Inc., Madison, WI.

Kastenbaum, M. A. and D. G. Hoel. 1970. Sample size requirements: one-way analysis of variance. Biometrika 57(2):421-430

Laskowski DA, Goring CAI, McCall PJ, & Swann RL. 1982. Terrestrial environment, pp. 198-240. In: RA Conway ed. Environmental Risk Analysis for Chemicals. Van Nostrand Reinhold Company, New York, NY.

Lichtenstein EP, Schulz KR, & Fuhremann TW. 1972. Movement and fate of dyfonate in soils under leaching and nonleaching conditions. J. Agr. Food Chem. 20:831-838.

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Lichtenstein EP, Fuhremann TW, Schulz KR, Liang TT. 1973. Effects of field application methods on the persistence and metabolism of phorate in soils and its translocation into crops. J. Econ. Entomol. 66:863-866.

Lichtenstein EP, Fuhremann TW, & Schulz KR. 1974. Translocation and metabolism of (^{14}C) phorate as affected by percolating water in a model soil-plant ecosystem. J. Agr. Food Chem. 22:991-996.

Malanchuk JL, Mueller CA, & Pomerantz SM. 1980. Microcosm evaluation of the agricultural potential of fly-ash-amended soils, pp. 1034-1049. In: JP Giesy, Jr. ed. Microcosms in Ecological Research, CONF-781101. U.S. Department of Energy, Technical Information Center.

Metcalf RL, Kapoor IP, Lu P, Schuth CK, & Sherman P. 1973. Model ecosystem studies of the environmental fate of six organochlorine pesticides. Environ. Hlth. Perspect. 4:35-44.

O'Neill RV, Ausmus BS, Jackson DR, Van Hook RI, Van Voris P, Washburne C, & Watson AP. 1977. Monitoring terrestrial ecosystems by analysis of nutrient export. Water Air Soil Pollut. 8:271-277.

Neter, J & Wasserman W. 1974. Applied linear statistical models, Irwin. Homewood, IL.

Parkinson, D, TRG Gray, ST Williams. 1971. Methods for Studying the Ecology of Soil Micro-organisms. IBP-Handbook No. 19. Blackwell Scientific Publications, Oxford, England. 115 p.

Phillipson, J. 1971. Methods of Study in Quantitative Soil Ecology, Population, Production and Energy Flow. IBP-Handbook No. 18. Blackwell Scientific Publications, Oxford, England. 297 p.

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Rand GM. 1980. Detection:bioassay. Pages 390-403. In: Guthrie FE & Perry JJ., eds. Introduction to Environmental Toxicology. Elsevier North Holland, Inc., N.Y. 484 pp.

Schindler JE, Waide JB, Waldron MC, Hains JJ, Schreiner SP, Freeman ML, Beng SL, Pettigrew DR, Schissel LA, & Clark PJ. 1980. A microcosm approach to the study of biogeochemical systems. I Theoretical rationale, pp. 192-203. In: JP Giesy, Jr. ed. Microcosms in Ecological Research, CONF-781101. U.S. Department of Energy, Technical Information Center.

Snedecor GW & Cochran WG. 1980. Statistical Methods. 7th Edition. Iowa State University Press, Ames, Iowa.

Sokal RR & Rohlf FJ. 1981. Biometry: The principles and practice of statistics in biological research 2nd Edition. W. H. Freeman and Company, San Francisco, CA.

Steele RDG & Torrie BH. 1980. Principals and Procedures of Statistics 2nd Edition. McGraw-Hill Book Co., N.Y. 481 pp.
Swann RL, Laskowski DA, McCall PJ, Vander Kuy K, & Dishburger HJ. 1983. A rapid method for the estimation of the environmental parameters octanol/water partition coefficient, soil sorption constant, water to air ratio, and water solubility. Residue Reviews. 85:17-28.

Tolle DA, Arthur MF, Van Voris P, Feder PI, Matthews MC, Chesson J, Larson ME, & Zwick TC. 1982. Evaluation of terrestrial microcosms for environmental studies of utility wastes. Third Ann. Prog. Rep. on RP 1224-5 to Electric Power Research Institute, Palo Alto, CA.

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Tolle DA, Arthur MF & Van Voris P. 1983. Microcosm/field comparison of trace element uptake in crops grown in fly ash-amended soil. Sci. Tot. Environ. 31: 243-261.

Van Voris P, O'Neill RV, Emanuel WR, & Shugart Jr, HH. 1980. Functional complexity and ecosystem stability. Ecology 61(6):1352-1360.

Van Voris P, Arthur MF, & Tolle DA. 1982. Evaluation of terrestrial microcosms for assessing ecological effects of utility wastes. EPRI Publication No. EA-2364. Electric Power Research Institute, Palo Alto, CA. pp. 178.

Van Voris P, Arthur MF, Tolle DA, Morris JP, & Larson M. 1983. Use of microcosms for monitoring nutrient cycling processes in agroecosystems. p. 171-182. In: R. Lowrance, R. Todd, L. Asmussen, and R. Leonard (eds.) Nutrient Cycling in Agricultural Ecosystems. University of Ga. College of Agric. Exp. Station, Spec. Pub. 23. Tifton, GA.

Van Voris P, Tolle DA, Arthur MF, Chesson J, & Zwick TC. 1984. Development and validation of terrestrial microcosm test system for assessing ecological effects of utility wastes. Final Project Report RP1224-5. EPRI Publication No. EA-3672. Electric Power Research Institute, Palo Alto, CA. pp. 185.

Van Voris P, Tolle DA, Arthur MF, & Chesson J. 1985. Terrestrial Microcosms: validation, applications, and cost-benefit analysis. pp 117-142 In: J Cairns, Jr. ed. Multispecies Toxicity Testing. Pergamon Press, New York.

Walters. DB. (ed) 1980. Safe Handling of Chemical Carcinogens, Mutagens, Teratogens and Highly Toxic Substances. Volumes I and II. Ann Arbor Science Publishers Inc., Ann Arbor Michigan. 652 pp.

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Wang, CH and DL Willis. 1965. Radiotracer Methodology in Biological Sciences. Prentice-Hall, Inc. Englewood Cliffs, New Jersey. 382 pp.

Zwick TC, Arthur MF, Tolle DA, & Van Voris P. 1984. A unique laboratory method for evaluating agro-ecosystem effects of an industrial waste product. Plant Soil. 77: 395-399.

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APPENDIX B

A noninclusive list of sample sources of biological materials for enclosed species tests

1. Ward's Natural Science Establishment, Inc.
   11850 E. Florence Ave.
   Santa Fe Springs, CA. 90670-4490

2. Harris Moran Seed Co.
   1155 Harkins Rd.
   Salinas, CA. 93901

3. Carolina Biological Supply Co.
   Burlington, North Carolina 27215

4. California Department Food and Agriculture
   Biological Control Services Program
   Sacramento, CA. 95832

Life-Cycle Characteristics of Non-Target Terrestrial Organisms for Testing Microbial Pest-Control Agents

Prepared by L.K. Etzel

System #1

A. *Amblyseius hibisci*

1. Length of life cycle:

   Egg - Egg: 11 days @ 25.5°C; 60% R.H.

2. Immature stage:

   Type of food: Pollen of oak, magnolia, cattail, *Mesembryanthemum* spp.; *Panonychus citri* (citrus red mite); *Oligonychus punicae* (brown almond mite)

   Quantity:

3. Adult:

   Type of food: Same

   Quantity: Ovipositing female eats 3 to 8 adult *O. punicae* per day

   Longevity: $\bar{X}$ ovipositional period = 19.6 days

   Fecundity: Ca. 2 eggs/female/day; Total ca. 40 eggs

4. Other notes:

B. *Panonychus citri*

1. Length of life cycle:

   Egg - Adult: 14 days @ 23.9°C; 65% R.H.; on lemon fruit
   33 days @ 15.6°C; 65% R.H.; on lemon fruit

2. Immature stage:

   Type of food: Citrus seedlings or lemon fruit

   Quantity:

3. Adult

   Type of food: Same

   Quantity:

   Longevity: 23 days @ 23.9°C; 65% R.H.; on lemon fruit
   36 days @ 15.6°C; 65% R.H.; on lemon fruit

   Fecundity: Total ca. 96 eggs @ 23.9°C as above
   Total ca. 50 eggs @ 15.6°C as above

4. Other notes:

C. References

1. Davis et al. (1979)

Life-Cycle Characteristics (con't)

System #1 (con't)

2. Kennett and Hamai (1980)
3. McMurtry (1970)
4. McMurtry and Johnson (1966)
5. McMurtry and Scriven (1964)
6. McMurtry and Scriven (1965)
7. McMurtry and Scriven (1966)
8. McMurtry, Huffaker, and van de Vrie (1970)
9. Munger (1963)
10. van de Vrie, McMurtry, and Huffaker (1972)

Life-Cycle Characteristics (con't)

System #2

A. Metaseiulus occidentalis

1. Length of life cycle:

   Egg - Adult: 6.3 days @ 24°C

2. Immature stage:

   Type of food: Tetranychus urticae (two-spotted mite)
   T. pacificus (Pacific spider mite)
   Various other Tetranychid mites

   Quantity: 16 - 17 mite eggs during development

3. Adult:

   Type of food: Same

   Quantity: Ovipositing female eats ca. 11 small two-spotted mite nymphs/day

   Longevity: $\bar{X}$ ovipositional period = 17.3 days

   Fecundity: Ca. 2 eggs/female/day; Total ca. 35 eggs

4. Other notes:

B. Tetranychus urticae

1. Length of life cycle:

   Egg - Egg: 19 days @ 20.3°C; 15.5 hr. light/day

2. Immature stage:

   Type of food: Attacks an extremely wide host range of plants

   Quantity:

3. Adult:

   Type of food: Same

   Quantity:

   Longevity: 18 days @ 20.3°C

   Fecundity: Ca. 2.4 eggs/female/day; Total ca. 38 eggs

4. Other notes:

C. References

(1) Davis et al. (1979)
(2) Kennett and Hamai (1980)
(6) McMurtry and Scriven (1965)
(8) McMurtry, Huffaker, and van de Vrie (1970)
(10) van de Vrie, McMurtry, and Huffaker (1972)
11. Iglinsky and Rainwater (1954)
12. Laing (1969)
(1971)

Life-Cycle Characteristics (con't)

System #3

A. Phytoseiulus persimilis

1. Length of life cycle:

Egg - Adult: 3.8 days @ 30°C
4.6 days @ 25°C
7.4 days @ 20°C

2. Immature stage:

Type of food: Tetranychus urticae (two-spotted spider mite)

Quantity:

3. Adult:

Type of food: Same

Quantity: Ovipositing female eats ca. 5 adult female two-spotted mites/day or ca. 34 mite eggs

Longevity: $\bar{X}$ ovipositional period ca. 14 days @ 26°C
Fecundity: Ca. 4 eggs/female/day; Total ca. 54 eggs

4. Other notes:

B. Tetranychus urticae

See info under System #2.

C. References

Same as for System #2.

Life-Cycle Characteristics (con't)

System #4

A. Geocoris punctipes (big-eyed bug)

1. Length of life cycle:

Egg - Adult: 34 days @ 26.7°C (incl. ca. 9 days in the egg stage)

2. Immature stage:

Type of food: Lygus bugs, spider mites, aphids, leafhoppers, and moth eggs and larvae

Quantity: 20 - 30 moth eggs/day/nymph

3. Adult:

Type of food: Same

Quantity: 50 - 60 moth eggs/day/adult

Longevity: Female ca. 68 days @ 25.5°C
Male ca. 42 days @ 25.5°C

Fecundity: Ca. 3.6 eggs/female/day; Total ca. 75 eggs @ 23.9°C (20% hatch)
Ca. 7.2 eggs/female/day; Total ca. 126 eggs @ 26.7°C (49% hatch)

4. Other notes:

B. Trichoplusia ni (cabbage looper)

1. Length of life cycle:

Egg - Adult: 20 - 21 days @ 28°C (incl. 13 - 14 days in the larval stage)

2. Immature stage:

Type of food: A variety of plants, including cabbage, turnip, lettuce, pea, and cotton; artificial diet.

Quantity:

3. Adult:

Type of food: Sugar water

Quantity:

Longevity: Ca. 8 days

Fecundity: $\overline{X}$ = ca. 400 eggs

4. Other notes:

C. References

14. Champlain and Sholdt (1967)
15. Cohen (1981)
16. Cohen (1985)
17. Cohen and Debolt (1983)

Life-Cycle Characteristics (con't)

System #4 (con't)

18. Dunbar and Bacon (1972a)
19. Dunbar and Bacon (1972b)
20. Guy et al.(1985)

Life-Cycle Characteristics (con't)

System #5

A. Chrysoperla carnea (green lacewing)

1. Length of life cycle:

   Egg - Adult: 24 - 27 days @ 26.7°C; 75% R.H. (incl. 15-16 days as larva)

2. Immature stage:

   Type of food: Principally aphids and moth eggs and young larvae

   Quantity: Ca. 30 - 40 mg moth eggs/larva; ca. 80 mg aphids/larva

3. Adult:

   Type of food: Honeydew, pollen; artificial diet

   Quantity:

   Longevity: 80 - 82 days @ 20°C & 80% R.H. with proper food

   Fecundity: Depends on food; artificial diet - ca. 890 eggs over 75 days; honey - 32 eggs over 46 days

4. Other notes: Rearing must be conducted with at least 14 hr. of light per day to prevent diapause.

B. Acyrthosiphon pisum (pea aphid)

1. Length of life cycle:

   Nymph - Nymph: 9 - 10 days @ 21°C

2. Immature stage:

   Type of food: Fava bean plants; alfalfa; other legumes

   Quantity:

3. Adult:

   Type of food: Same

   Quantity:

   Longevity: Estimate 10 days

   Fecundity: Ca. 9 nymphs/female/day; ca. 90 nymphs total

4. Other notes:

C. References:

(1) Davis et al. (1979)
21. Canard et al. (1984)
22. Forbes et al. (1985)
23. Morrison (1985a)

Life-Cycle Characteristics (con't)

System #6

A. *Hippodamia convergens* (convergent ladybird beetle)

1. Length of life cycle:

   Egg - Adult: 17 days @ 26°C (incl. ca. 9 days as larva)

2. Immature stage:

   Type of food: Aphids

   Quantity: Estimate 15 mg/larva/day; or 140 - 170 mg/larval stage

3. Adult:

   Type of food: Same

   Quantity: Estimate 25 mg/adult/day; or ca. 1350 mg/adult stage

   Fecundity: Ca. 1270 eggs; ca. 25 eggs/female/day

   Longevity: $\bar{X}$ = 60 days @ 22.8°C

4. Other notes: Rearing should be conducted with at least 11 hours of light.

B. *Acyrthosiphon pisum*

See info under System #5.

C. References:

(1) Davis et al. (1979)
(22) Forbes et al. (1985)
24. Clausen (1916)
25. Hagen (1962)
26. Hagen and Sluss (1966)
27. Simpson and Burkhardt (1960)

Life-Cycle Characteristics (con't)

System #7

A. Olla abdominalis (ashy-gray ladybird beetle)

1. Length of life cycle:

   Egg - Adult: 15 days @ 26°C (incl. 8 days as larva)

2. Immature stage:

   Type of food: Aphids

   Quantity: Estimate 15 mg/larva/day; or 125 mg/larval stage

3. Adult:

   Type of food: Same

   Quantity: Estimate 15 mg/adult/day; or ca. 675 mg/adult stage

   Longevity: Estimate 45 days

   Fecundity: Ca. 295 eggs; ca. 8 eggs/female/day

4. Other notes:

B. Acyrthosiphon pisum

See info under System #5.

C. References:

(1) Davis et al. (1979)
(22) Forbes et al. (1985)
(24) Clausen
(25) Hagen
(27) Simpson and Burkhardt (1960)

Life-Cycle Characteristics (con't)

System #8

A. Cryptolaemus montrouzieri (mealybug destroyer)

1. Length of life cycle:

Egg - Adult: 28 - 29 days @ 26.7°C (incl. ca. 15 days as larva)
43 - 47 days @ 21.1°C (incl. ca. 25 days as larva)

2. Immature stage:

Type of food: Mealybugs

Quantity:

3. Adult:

Type of food: Same

Quantity:

Longevity: $\bar{X}$ = 51 days @ 25°C

Fecundity: Ca. 440 eggs; ca. 9 eggs/female/day @ 25°C

4. Other Notes:

B. Pseudococcus citri (citrus mealybug)

1. Length of life cycle:

Egg - Adult: Estimate 4-5 weeks @ ca. 21.1°C

2. Immature stage:

Type of food: Wide host range of plants; especially severe on citrus

Quantity:

3. Adult:

Type of food: Same

Quantity:

Longevity: Estimate 4 weeks

Fecundity: Ca. 300 - 500 eggs

4. Other notes:

C. References:

28. Fisher (1963)

Life-Cycle Characteristics (con't)

System #9

A. *Trichogramma* spp. (parasites of insect eggs)

1. Length of life cycle:

   Egg - Adult: 8 - 10 days @ 26.7°C; 80% R.H.; continuous light

2. Immature stage:

   Type of food: Primarily attack eggs of lepidopterans

   Quantity: 1 - 2 parasites/egg

3. Adult:

   Type of food: Honey

   Quantity:

   Longevity:

   Fecundity: Less than 100 eggs

4. Other notes:

B. *Ostrinia nubilalis* (European corn borer)

1. Length of life cycle:

   Egg - Adult: 21 - 28 days @ 27°C; 75 - 80% R.H.; continuous light (incl. 13 - 19 days as larva)

2. Immature stage:

   Type of food: Wide host range, including corn, potatoes, beans, celery, etc.; artificial diet

   Quantity:

3. Adult:

   Type of food: Water

   Quantity:

   Longevity: 12 days

   Fecundity: $\bar{X}$ = 300 eggs/female

4. Other notes:

C. References:

29. Burbutis and Goldstein (1983)
30. Guthrie et al. (1985)
31. Morrison (1985b)

Life-Cycle Characteristics (con't)

System #10

A. Anaphes flavipes (egg parasite of cereal leaf beetle)

1. Length of life cycle:

   Egg - Adult: 8 - 9 days @ 30°C; 80+% R.H.

2. Immature stage:

   Type of food: Eggs of cereal leaf beetle

   Quantity: 3+ parasites/egg

3. Adult:

   Type of food: Honey

   Quantity:

   Longevity: 4 - 5 days @ 21°C; 80+% R.H.

   Fecundity: Ca. 21 eggs/female

4. Other notes:

B. Oulema melanopus (cereal leaf beetle)

1. Length of life cycle:

   Egg - Adult:

2. Immature stage:

   Type of food:

   Quantity:

3. Adult:

   Type of food:

   Quantity:

   Longevity:

   Fecundity:

4. Other notes:

C. References:

32. Anderson and Paschke (1968)

Life-Cycle Characteristics (con't)

System #11

A. Aphidius ervi (parasite of pea aphid)

1. Length of life cycle:

Egg - Adult: Ca. 14 days @ 21.1°C; 55% R.H.

2. Immature stage:

Type of food: Pea aphids

Quantity: 1 parasite/aphid

3. Adult:

Type of food: Honeydew or honey

Quantity:

Longevity: 8 - 10 days @ 21.1°C

Fecundity: Ca. 50 adult progeny/female with large-scale rearing

4. Other notes:

B. Acyrthosiphon pisum

See info under System #5.

C. References:

(22) Forbes et al. (1985)
33. Fox et al. (1967)
34. Quezada (unpubl.)

Life-Cycle Characteristics (con't)

System #12

A. Diaretiella rapae (parasite of green peach aphid and cabbage aphid)

1. Length of life cycle:

Egg - Adult: 10.4 days @ 25°C
14.7 days @ 20°C
20.2 days @ 15°C

2. Immature stage:

Type of food: Green peach aphids or cabbage aphids

Quantity: 1 parasite/aphid

3. Adult:

Type of food: Honeydew or honey

Quantity:

Longevity: Maximum of 6 days @ ca. 19°C

Fecundity: Ca. 230 progeny/female

4. Other notes:

B. Brevicorynae brassicae (cabbage aphid)

1. Length of life cycle:

Nymph - Nymph: Estimate 10 - 14 days @ 21.1°C

2. Immature stage:

Type of food: Wide variety of cole plants, including cabbage, collards, and rape

Quantity:

3. Adult:

Type of food: Same

Quantity:

Longevity: Ca. 30 days in nature in summer

Fecundity: Ca. 80 - 100 nymphs

4. Other notes:

C. References:

(22) Forbes et al. (1985)
35. Hagen and van den Bosch (1968)
36. Simpson et al. (1975)

Life-Cycle Characteristics (con't)

System #13

A. *Lysiphlebus testaceipes* (parasite of greenbug)

1. Length of life cycle:

   Egg - Adult: 11.1 days @ 21 - 32°C
   10.8 days @ 27°C
   13.2 days @ 21°C

2. Immature stage:

   Type of food: Greenbugs

   Quantity: 1 parasite/aphid

3. Adult:

   Type of food: Honeydew or honey

   Quantity:

   Longevity: 2 - 3 days @ 23.3°C

   Fecundity:

4. Other notes: The parasite is not active at temperatures below 18.3°C, but the greenbug can reproduce at temperatures as low as 4.4°C.

B. *Schizaphis graminum* (greenbug)

1. Length of life cycle:

   Nymph - Nymph: 6 days @ 26.7°C
   9 days @ 21.1°C
   15 days @ 15.6°C

2. Immature stage:

   Type of food: Wheat, barley, oats, rye, and sorghum

   Quantity:

3. Adult:

   Type of food: Same

   Quantity:

   Longevity: Ca. 20 days @ 22.2°C

   Fecundity: Ca. 100 nymphs/female

4. Other notes:

C. References:

(22) Forbes et al. (1985)
37. Headlee (1914)
38. Hight et al. (1972)
39. Starks and Burton (1977)

Life-Cycle Characteristics (con't)

System #14

A. Cotesia (=Apanteles) melanoscelus (parasite of gypsy moth)

1. Length of life cycle:

   Egg - Adult: Ca. 15 days @ 23°C; 40% R.H.; 18 hr. light daily

2. Immature stage:

   Type of food: Larvae of gypsy moths

   Quantity: 1 parasite/larva

3. Adult:

   Type of food: Honey

   Quantity:

   Longevity: At least 2 - 3 weeks

   Fecundity: Ca. 100 progeny/female

4. Other notes:

B. Lymantria dispar (gypsy moth)

1. Length of life cycle:

   Egg - Adult: Ca. 40 - 44 days @ 25°C; 50 - 60% R.H.; 16 hr. light daily

2. Immature stage;

   Type of food: Foliage of over 300 species of trees and shrubs, expecially oaks; artificial diet

   Quantity: Ca. 10 ml diet per larva

3. Adult:

   Type of food: None

   Quantity: N/A

   Longevity: Males = 3 - 5 days; females = 2 - 3 days

   Fecundity: Ca. 900 eggs/female

4. Other notes:

C. References:

40. Chianese (1985)
41. ODell et al. (1985)

Life-Cycle Characteristics (con't)

System #15

A. Metaphycus helvolus (parasite of black scale)

1. Length of life cycle:

   Egg - Adult: 13 days @ 27.8°C

2. Immature stage:

   Type of food: Black scale

   Quantity: 1 parasite/scale

3. Adult:

   Type of food: Honey

   Quantity:

   Longevity: 50 days @ 26.7°C, if fed honey; but can live much longer with cool temperatures and host feeding

   Fecundity: Ca. 400 eggs/female

4. Other notes:

B. Saissetia oleae (black scale)

1. Length of life cycle:

   Egg - Adult: 70 - 90 days @ 21°C

2. Immature stage:

   Type of food: Wide host plant range among the ornamentals; can be severe pest of citrus and olive.

   Quantity:

3. Adult:

   Type of food: Same

   Quantity:

   Longevity: Estimate 1 month @ 21°C

   Fecundity: 1,000 - 4,000 eggs

4. Other notes:

C. References:

42. Clausen (1978)
43. Flanders (1942)

Life-Cycle Characteristics (con't)

System #16

A. Aphytis lingnanensis (parasite of California red scale)

1. Length of life cycle:

Egg - Adult: Ca. 15 days @ 27°C; 60% R.H.
17 days @ 26°C; 60% R.H.

2. Immature stage:

Type of food: California red scale; oleander scale (in lab)

Quantity: 2 or more parasites/scale

3. Adult:

Type of food: Honey

Quantity:

Longevity: Ca. 10 days @ 26°C; 60% R.H.

Fecundity: Ca. 5 - 6 eggs/day; ca. 57 eggs/female total on red scale; more than twice this number on oleander scale; each female destroys ca. an additional 46 scales by predation

4. Other notes:

B. Aspidiotis nerii (oleander scale)

1. Length of life cycle:

Crawler - Crawler: 60 days @ 25°C; 50 - 60% R.H.

2. Immature stage:

Type of food: Oleander plants; banana squash or butternut pumpkins

Quantity:

3. Adult:

Type of food: Same

Quantity:

Longevity: 10 - 14 days

Fedundity: $\bar{X}$ = 94 crawlers/female

4. Other notes:

C. References:

44. DeBach and White (1960)
45. Papacek and Smith (1985)

Life-Cycle Characteristics (con't)

System #17

A. Brachymeria intermedia (parasite of gypsy moth and many other lepidopterans)

1. Length of life cycle:

   Egg - Adult: 15 - 30 days @ 24°C

2. Immature stage:

   Type of food: Larvae in several lepidopterous families

   Quantity: 1 parasite/larva

3. Adult:

   Type of food: Honey

   Quantity:

   Longevity: 4 - 6 weeks @ 24° - 27°C under forced lab prod.; 150 days @ 21° - 24°C with honey and water and no hosts

   Fecundity: Ca. 300 progeny/female

4. Other notes:

B. Trichoplusia ni (cabbage looper)

See info under System #4.

C. References:

(20) Guy et al. (1985)
46. King and Hartley (1985a)
47. Palmer (1985)

Life-Cycle Characteristics (con't)

System #18

A. Lixophaga diatraeae (fly parasite on sugarcane borer)

1. Length of life cycle:

Egg - Adult: 18.2 - 20.9 days @ 26°C; 80% R.H.

2. Immature stage:

Type of food: Larvae of the sugarcane borer and of the greater wax moth (lab only)

Quantity: 1 parasite/larva

3. Adult:

Type of food: Raw brown sugar

Quantity:

Longevity: Female = ca. 24 days @ 26°C
Male = ca. 30 days @ 26°C

Fecundity: Peak fecundity = 78 maggots in uterus on 12th day

4. Other notes:

B. Diatraea saccharalis (sugarcane borer)

1. Length of life cycle:

Egg - Adult: 36 - 38 days @ 26°C

2. Immature stage:

Type of food: Sugarcane or grain sorghum; artificial diet in lab

Quantity: Artificial diet - ca. 3.3 ml/larva
Grain sorghum stalk - 1.5"/larva
Sugarcane tops - ca. 15 gm/larva

3. Adult:

Type of food: Water

Quantity:

Longevity: Ca. 7 days @ 26°C

Fecundity: 435 eggs/female @ 26°C
730 eggs/female @ 24°C

4. Other notes:

C. References:

(46) King and Hartley (1985a)
48. King and Hartley (1985b)
49. King and Hartley (1985c)
50. King and Martin (1975)

Life-Cycle Characteristics (con't)

System #18 (con't)

51. King et al. (1975)
52. King et al. (1975)
53. King et al. (1979)
54. Miles and King (1975)
55. Pan and Long (1961)
56. Wongsiri and Randolph (1962)

Life-Cycle Characteristics (con't)

System #19

A. *Apis mellifera* (honeybee)

1. Adult:

Type of food: Honey, pollen, and water

Quantity:

Longevity: Foragers live ca. 3 weeks in nature; what would happen in a caged environment is unpredictable. Freshly emerged bees from the brood comb should live the longest (at least a month in the proper conditions) but may need to be fed pollen in addition to honey. There should be at least 50 bees placed in a microcosm so they can aggregate to maintain suitable environmental conditions.

Life-Cycle Characteristics (con't)

System #20

A. *Vanessa cardui* (Painted lady butterfly)

1. Length of life cycle:

Egg - Adult: Egg - ca. 1 week in nature in summer
Larva - ca. 1 month in nature in summer
Pupa - ca. 2 weeks in nature in summer

Total - ca. 7 weeks

2. Immature stage:

Type of food: Can feed on over 100 spp. of plants (chiefly in the Malvaceae, Compositae, Leguminosae); plants include thistles (a favorite), burdock, sunflowers, nettles; can be a serious pest at times on maize, alfalfa, beans, sunflowers, and soybeans. Artificial diets available.

Quantity:

3. Adult:

Type of food:

Quantity:

Longevity: No info found, but should be quite long-lived because of potential for migration.

Fecundity:

4. Other notes:

B. References:

57. Brooks and Knight (1982)
58. Singh (1985)
59. Williams, C.B. (1970)

Proposed Non-Target Terrestrial Organisms
for Testing Microbial Pest Control Agents

(Revised Bibliography)

Prepared by L.K. Etzel

I. General References

A. Bezark, L.G. and H. Yee
1985. 1985 Suppliers of Beneficial Organisms in North America. California Department of Food and Agriculture, Biological Control Services Program, 3288 Meadowview Road, Sacramento, CA 95832. 6 pp.

B. Dickerson, W.A., J.D. Hoffman, E.G. King, N.C. Leppla, and T.M. ODell
1979. Arthropod Species in Culture in the United States and Other Countries. Entomological Society of America, College Park, MD. 93 pp.

C. Singh, P. and R.F. Moore (eds.)
1985. Handbook of Insect Rearing. Volumes I and II. Elsevier, New York. 488 pp. & 514 pp.

II. Specific References

System #1

1. Davis, D.W., S.C. Hoyt, J.A. McMurtry, and M.T. AliNiazee (eds.)
1979. Biological Control and Insect Pest Management. University of California Press, Berkeley. 102 pp.

2. Kennett, C.E. and J. Hamai
1980. Oviposition and development in predaceous mites fed with artificial and natural diets (Acari:Phytoseiidae). Ent. Exp. Appl., 28:116-122.

3. McMurtry, J.A.
1970. Some factors of foliage condition limiting population growth of Oligonychus punicae (Acarina:Tetranychidae). Ann. Entomol. Soc. Amer., 63:406-412.

4. McMurtry, J.A. and H.G. Johnson
1966. An ecological study of the spider mite Oligonychus punicae (Hirst) and its natural enemies. Hilgardia, 37:363-400.

5. McMurtry, J.A. and G.T. Scriven
1964. Studies on the feeding, reproduction, and development of Amblyseius hibisci (Acarina:Phytoseiidae) on various food substances. Ann. Entomol. Soc. Amer., 57:649-655.

6. McMurtry, J.A. and G.T. Scriven
1965. Insectary production of phytoseiid mites. Jour. Econ. Entomol., 58:282-284.

7. McMurtry, J.A. and G.T. Scriven
1966. The influence of pollen and prey density on the number of prey consumed by Amblyseius hibisci (Acarina:Phytoseiidae). Ann. Entomol. Soc. Amer., 59:147-149.

8. McMurtry, J.A., C.B. Huffaker, and M. van de Vrie
1970. Ecology of Tetranychid mites and their natural enemies: A review. I. Tetranychid enemies: Their biological characters and the impact of spray practices. Hilgardia, 40:331-390.

9. Munger, F.
1963. Factors affecting growth and multiplication of the citrus red mite, Panonychus citri. Ann. Entomol. Soc. Am., 56:867-874.

10. van de Vrie, M., J.A. McMurtry, and C.B. Huffaker
1972. Ecology of Tetranychid mites and their natural enemies: A review. III. Biology, ecology, and pest status, and host-plant relations of tetranychids. Hilgardia, 41:343-432.

System #2

References 1, 2, 6, 8, 10

11. Iglinsky, W., Jr., and C.F. Rainwater
1954. Life history and habits of Tetranychus desertorum and bimaculatus on cotton. Jour. Econ. Entomol., 47:1084-1086.

12. Laing, J.E.
1969. Life history and life table of Tetranychus urticae Koch. Acarologia, 9:32-42.

13. Scriven, G.T. and J.A. McMurtry
1971. Quantitative production and processing of Tetranychid mites for large-scale testing or predator production. Jour. Econ. Entomol., 64:1255-1257.

System #3

References 1, 2, 6, 8, 10, 11, 12, 13

System #4

14. Champlain, R.A. and L.L. Sholdt
1967. Life history of Geocoris punctipes (Hemiptera:Lygaeidae) in the laboratory. Annals Entomol. Soc. Amer., 60:881-883.

15. Cohen, A.C.
1981. An artificial diet for Geocoris punctipes (Say). Southwest. Entomol., 6:109-113.

16. Cohen, A.C.
1985. Simple method for rearing the insect predator Geocoris punctipes (Heteroptera:Lygaeidae) on a meat diet. Jour. Econ. Entomol., 78:1173-1175.

17. Cohen, A.C. and J.W. DeBolt
1983. Rearing Geocoris punctipes on insect eggs. Southwest. Entomol., 8:61-64.

18. Dunbar, D.M. and O.G. Bacon
1972a. Feeding, development, and reproduction of Geocoris punctipes (Heteroptera:Lygaeidae) on eight diets. Ann. Entomol. Soc. Amer., 65:892-895.

19. Dunbar, D.M. and O.G. Bacon
1972b. Influence of temperature on development and reproduction of Geocoris atricolor, G. pallens, and G. punctipes (Heteroptera: Lygaeidae) from California. Envir. Entomol., 1:596-599.

20. Guy, R.H., N.C. Leppla, and J.R. Rye
1985. Trichoplusia ni. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 487-494. Elsevier, New York, 514 pp.

System #5

Reference 1

21. Canard, M. Y. Semeria, and T.R. New (eds.)
1984. Biology of Chrysopidae. Dr W. Junk Publishers, Boston. 294 pp.

22. Forbes, A.R., B.D. Frazer, and C.K. Chan
1985. Aphids. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 353-359. Elsevier, New York, 488 pp.

23. Morrison, R.K.
1985a. Chrysopa carnea. In "Handbook of Insect Rearing" (P.Singh and R.F. Moore, eds.), Vol. I, pp. 419-426, Elsevier, New York, 488 pp.

System #6

References 1, 22

24. Clausen, C.P.
1916. Life-history and feeding records of a series of California Coccinellidae. Calif. Univ. Pub. Entomol., 1:251-299.

25. Hagen, K.S.
1962. Biology and ecology of predaceous Coccinellidae. Annu. Rev. Entomol., 7:289-326.

26. Hagen, K.S. and R.R. Sluss
1966. Quantity of aphids required for reproduction by Hippodamia spp. in the laboratory. Pp. 47-59. In "Ecology of Aphidophagous Insects" (I. Hodek, ed.), Dr. W. Junk, Publ., The Hague, Netherlands, 360 pp.

27. Simpson, R.G. and C.C. Burkhardt
1960. Biology and evaluation of certain predators of Therioaphis maculata (Buckton). Jour. Econ. Entomol., 53:89-94.

System #7

References 1, 22, 24, 25, 27

System #8

28. Fisher, T.W.
1963. Mass culture of Cryptolaemus and Leptomastix, natural enemies of citrus mealybug. Calif. Agr. Expt. Stn. Bull., 797:39.

System #9

29. Burbutis, P.P. and L.F. Goldstein
1983. Mass rearing Trichogramma nubilale on European corn borer, its natural host. Protection Ecology, 5:269-275.

30. Guthrie, W.D., J.C. Robbins, and J.L. Jarvis
1985. Ostrinia nubilalis. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 407-413. Elsevier, New York, 514 pp.

31. Morrison, R.K.
1985b. Trichogramma spp. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 413-417, Elsevier, New York. 488 pp.

System #10

32. Anderson, R.C. and J.D. Paschke
1968. The biology and ecology of Anaphes flavipes (Hymenoptera:Mymaridae), an exotic egg parasite of the cereal leaf beetle. Ann. Entomol. Soc. Amer., 61:1-5.

System #11

Reference 22

33. Fox, P.M., B.C. Pass, and R. Thurston
1967. Laboratory studies on the rearing of Aphidius smithi (Hymenoptera: Braconidae) and its parasitism of Acyrthosiphon pisum (Homoptera: Aphididae). Ann. Entomol. Soc. Amer., 60:1083-1087.

34. Quezada, J.
unpubl. Notes on rearing Acyrthosiphon pisum and Aphidius ervi.

System #12

Reference 22

35. Hagen, K.S. and R. van den Bosch
1968. Impact of pathogens, parasites, and predators on aphids. Annu. Rev. Entomol., 13:325-384.

36. Simpson, B.A., W.A. Shands, and G.W. Simpson
1975. Mass rearing of the parasites Praon sp. and Diaretiella rapae. Ann. Entomol. Soc. Am., 68:257-260.

System #13

Reference 22

37. Headlee, T.J.
1914. Some data on the effect of temperature and moisture on the rate of insect metabolism. Jour. Econ. Entomol., 7:413-417.

38. Hight, S.C., R.D. Eikenbary, R.J. Miller, and K.J. Starks
1972. The greenbug and Lysiphlebus testaceipes. Environ. Entomol., 1:205-209.

39. Starks, K.J. and R.L. Burton
1977. Greenbugs: determining biotypes. USDA-ARS Tech. Bull., 1556:12.

System #14

40. Chianese, R.
1985. Cotesia melanoscelus. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 395-400, Elsevier, New York, 488 pp.

41. ODell, T.M., C.A. Butt, and A.W. Bridgeforth
1985. Lymantria dispar. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 355-367, Elsevier, New York, 514 pp.

System #15

42. Clausen, C.P. (ed.)
1978. Introduced parasites and predators of arthropod pests and weeds: A world review. USDA Agric. Handbook No. 480, 545 pp.

43. Flanders, S.E.
1942. Metaphycus helvolus, an encyrtid parasite of the black scale. Jour. Econ. Entomol., 35:690-698.

System #16

44. DeBach, P. and E.B. White
1960. Commercial mass culture of the California red scale parasite Aphytis lingnanensis. Calif. Agric. Exp. Stn. Bull. No. 770, 58pp.

45. Papacek, D.F. and D. Smith
1985. Aphytis lingnanensis. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 373-381, Elsevier, New York, 488 pp.

System #17

Reference 20

46. King, E.G. and G.G. Hartley
1985a. Galleria mellonella. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 301-305, Elsevier, New York, 514 pp.

47. Palmer, D.J.
1985. Brachymeria intermedia. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. I, pp. 383-393, Elsevier, New York, 488 pp.

System #18

Reference 46

48. King, E.G. and G.G. Hartley
1985b. Lixophaga diatraeae. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 119-123, Elsevier, New York, 514 pp.

49. King, E.G. and G.G. Hartley
1985c. Diatraea saccharalis. In "Handbook of Insect Rearing" (P. Singh and R.F. Moore, eds.), Vol. II, pp. 265-270, Elsevier, New York, 514 pp.

50. King, E.G. and D.F. Martin
1975. Lixophaga diatraeae: Development at different constant temperatures. Environ. Entomol., 4:329-332.

51. King, E.G., F.D. Brewer, and D.F. Martin
1975. Development of Diatraea saccharalis (Lep.:Pyralidae) at constant temperatures. Entomophaga, 20:301-306.

52. King, E.G., D.F. Martin, and L.R. Miles
1975. Advances in rearing of Lixophaga diatraeae (Dipt.: Tachinidae). Entomophaga, 20:307-311.

53. King, E.G., G.G. Hartley, D.F. Martin, J.W. Smith, T.E. Summers, and R.D. Jackson
1979. Production of the Tachinid Lixophaga diatraeae on its natural host, the sugarcane borer, and on an unnatural host, the greater wax moth. U.S. Dept. of Agric., SEA, AAT-S-3/April 1979.

54. Miles, L.R. and E.G. King
1975. Development of the Tachinid parasite, Lixophaga diatraeae, on various developmental stages of the sugarcane borer in the laboratory. Environ. Entomol., 4:811-814.

55. Pan, Yung-Song and W.H. Long
1961. Diets for rearing the sugarcane borer. Jour. Econ. Entomol., 54:257-261.

56. Wongsiri, T. and N.M. Randolph
1962. A comparison of the biology of the sugarcane borer on artificial and natural diets. Jour. Econ. Entomol., 55:472-473.

System #19

No references.

System #20

57. Brooks, M. and C. Knight
1982. A Complete Guide to British Butterflies. Jonathan Cape, London. 159 pp.

58. Singh, P.
1985. Multiple-species rearing diets. In "Handbook of Insect Rearing" (P. Singh & R.F. Moore, eds.), Vol. I, pp. 19-44, Elsevier, New York, 488 pp.

59. Williams, C.B.
1970. The migration of the painted lady butterfly, Vanessa cardui (Nymphalidae), with special reference to North America. J. Lepid. Soc., 24:157-175.

PATHOLOGY-MICROBIOLOGY SUBGROUP

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Pathology - Microbiology Subgroup Critique and Recommendations

This subgroup was formed to facilitate adaptation of the terrestrial, freshwater and estuarine enclosed systems to testing microbial pesticides. This charge was fulfilled through evaluation of the systems and by providing background information to the other subgroups on the properties, pathology, taxonomy and ecology of the various groups of agents that are the active components of microbial pesticides (Federal Register, 1984). The objective was to ensure, as far as was possible, that the enclosed systems resulting from the workshop were (1) compatible with the biological, chemical and physical properties of the agents, and (2) the endpoints measured in the systems were appropriate for infectious and toxic living agents. The micro-biology-pathology subgroup members participated in the discussions of the other subgroups concerned with the design, function and composition of the enclosed systems.

The following is a summary of our critique and recommendations. A concensus developed among members of the subgroup during the course of the workshop that a common fault with most of the enclosed systems was that they were developed for environmental chemical toxicants and microbial agents have not been tested with them. There is concern that the endpoints and parameters measured in some of them may possibly be inappropriate for microbial agents or may be too insensitive for the generally milder and more specific effects of microbial pesticidal agents. Because of the use for which they were originally intended, some of the design features and conditions of the enclosed systems may either be incompatible with or disadvantageous to the microbial agents because their tolerance limits are usually narrower than those of chemical toxicants. Consequently, their full potential effect may not be realized in the enclosed system. As an

example, during discussions of the freshwater subgroup it was found that the diurnal variation in pH in the aquatic littoral microcosm can attain alkalinities in the range which affect viral occlusion bodies (Edgawa and Summers, 1972; Kawanishi, et al., 1972) and certain bacterial pesticide protoxin crystals (Cooksey, 1971). Thus, the effects observed with these agents in this system may be dependent on when during the diurnal cycle the pesticidal agent is introduced. Closer examination of other enclosed systems may reveal analogous situations which must be circumvented to effectively utilize them for ecological assessments.

The following are the recommendations of the committee: (1) The compatibility of each enclosed system and the specific properties of the agent being carefully examined on a case-by-case basis before it is recommended for testing. The basis for this recommendation are the following considerations. Most systems were originally developed for toxicity testing and not designed specifically with microbial pesticides in mind. Additionally, the subgroup can neither anticipate the diverse types of microbial agents nor the variety of modes of action to be tested in these systems. Frequently pesticidal action is accomplished through infectivity and pathogenicity (Payne, 1982). Even where toxicity is the mode of action, both the nature of the toxins (Wilcox et al., 1986) as well as the mechanisms of action (Luthy and Ebersold, 1981) appear to be different from most environmental chemical toxicants. Additionally, a single microbial pesticide may have multiple modes of action such as a combination of infectivity and toxicity and one action may be dependent upon the other (Heimpel and Angus, 1963). Also, because the systems were designed to test the relatively severe effects frequently manifested by chemical pesticide toxicity, their utility with microbial pesticidal agents which may not act through acute or lethal

effects but rather through chronic and debilitating action resulting in reduced vigor and fecundity of the affected species (e.g., Katagiri, 1981) in question; (2) Where possible positive controls should be included; (3) The systems should eventually be validated with microorganisms of known effects.

References

Cooksey, K.E. 1971. The protein crystal toxin of Bacillus thuringiensis: biochemistry and mode of action, p.247-274. In H.D. Burges and H.W. Hussey (ed.), Microbial Control of Insects and Mites. Academic Presss, Inc., New York.

Egawa, K. and Summers, M.D. 1972. Solubilization of Trichoplusia ni granulosis virus proteinic crystal I. Kinetics. J. Invertebr. Pathol. 19, 395-404.

Fereral Register. 1984. Data Requirements for Pesticide Registration. Vol. 49, 42856-42905.

Heimpel, A.M. and Angus, T.A. 1963. Diseases caused by sporeforming bacteria, p.42. In Steinhaus, E.A. (ed.), Insect Pathology - An Advanced Treatise. Academic Press, New York and London.

Katagiri, K. 1981. Pest control by cytoplasmic polyhedrosis viruses, p. 433-440. In Burges, H.D. (ed.), Microbial Control of Pests and Plant Diseases 1970-1980. Academic Press, London, New York, Toronto, Sidney, San Francisco.

Kawanishi, C.Y. Egawa, K. and Summers, M.D. 1972. Solubilization of Trichoplusia ni granulosis virus proteinic crystal II. Ultrastructure. J. Invertebr. Pathol. 20, 95-100.

Luthy, P. and Ebersold, H.R. 1981. Bacillus thuringiensis delta-endotoxin: Histopathology and molecular mode of action, p. 235-267. In E.W. Davidson (ed.), Pathogenesis of Invertebrate Microbial Diseases. Allanheld, Osmun & Co. Publishers, Inc.

Payne, C.C. 1982. Insect viruses as control agents. Parasitol. 84, 35-77.

Wilcox, D.R., Shivakumar, A.G., Melin, B.E., Miller, M.F., Benson, T.A., Schopp, C.W., Casuto, D., Gundling, G.J., Bolling, T.J., Spear, B.B. and Fox, J.L. 1986. Genetic Engineering of Bio-Insecticides. In Protein Engineering: Applications in Science, Industry and Medicine (In press).

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