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Project Summary

An Enclosed Aquatic Multispecies Test System for Testing Microbial Pest Control Agents with Non-Target Species

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An enclosed test system was developed in which multiple species of aquatic animals and plants were tested experimentally for adverse non-target effects of wild-type and genetically altered microbial pest control agents (MPCAs). The test system consisted of components that were inexpensive and readily available from aquaculture supply companies, pet shops, and building material stores. A variety of marine and freshwater non-target animal and plant species (NTOs) representing diverse phylogenetic taxa and trophic levels, were collected from wild populations or purchased from commercial suppliers.

Four different types of model MPCAs were tested in the multispecies system. These included two different strains of the mosquito pathogen *Bacillus sphaericus*, a strain of *Pseudomonas putida* (used as a model for the genus), and the insect baculovirus AcMNPV. The fate, persistence, and infectivity of these model MPCAs were evaluated experimentally using traditional microbiological and histological methods. Also used were assays specific for the model MPCAs that had been altered by addition of a unique genetic marker. For two of the model MPCAs, gene probes were used as a detection method to track the MPCA in the test system water and NTOs.

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

Introduction

Microbial pest control agents (MPCAs), also known as biological control agents or "biorationals", are microbial agents intended for use in controlling detrimental insects, weeds, and other pests. Because they may be applied in rather large quantities or repeatedly applied in smaller quantities to areas outside the normal geographic range of the wild type pathogen, it is important that test data be obtained prior to field application. This data will help to predict the fate and persistence of MPCAs in the environment and their effect on non-target organisms that would be exposed as a result of normal field application. Acquisition of such information becomes even more important when the application of genetically altered MPCAs is considered.

The purpose of the project reported here was to develop a simple, functionally closed aquatic multispecies test system in which the study of MPCAs could be accomplished in a manner that models an ecosystem and utilizes as many different, yet readily available types of NTOs as possible.

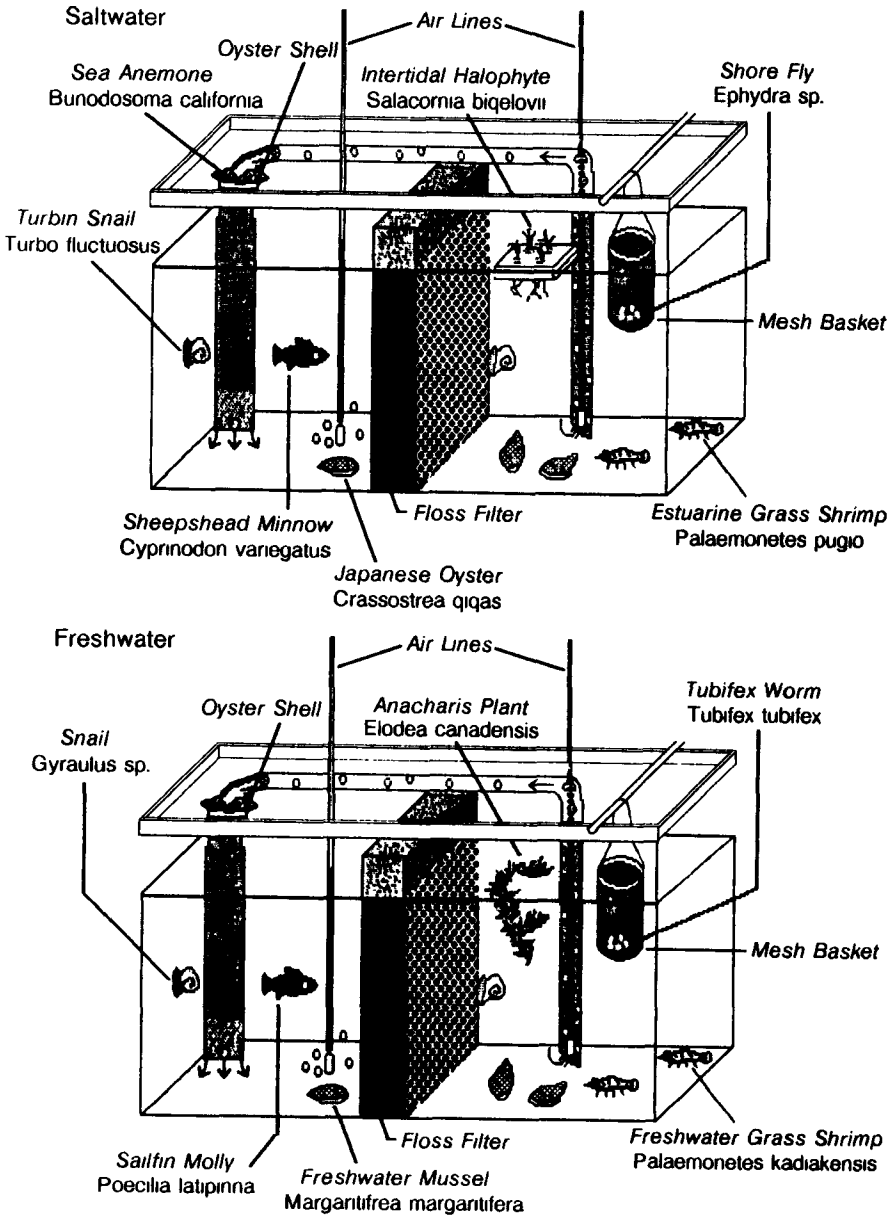
Experimental

Test System

The test system consisted of components that were inexpensive and readily available from a variety of commercial suppliers including aquaculture supply companies, pet shops, and building supply stores. Specifically, the system consisted of a standard 120 L glass aquarium that contained a vertical biological filter. This vertical biological filter also served as a barrier, functioning to divide the tank in two halves and to provide a physical barrier between populations of experimental animals so as to prevent unplanned inter specific predation. The biological filter matrix consisted of a polyester fiber pad. An obvious advantage of such vertical highly porous pad-type biological filters over traditional undergravel filters is that they do not remove suspended particulates (such as an introduced MPCA and algal food organisms) as rapidly as do undergravel filters. The pH of water in tanks with polyester pad filters was maintained by suspending 1 L plastic beakers with perforated bottoms and half filled with crushed oyster shell or dolomite under the outlet of an airlift pump (Figures 1 and 2).

MPCA Tests and Non-Target Test Organisms

A variety of marine, estuarine and freshwater vertebrate and invertebrate animals and plants were acquired for potential use as non-target test organisms (NTOs) in model MPCA tests. The selection criteria for potential NTOs were that they should be common and readily available from cultured laboratory stocks, commercial suppliers, or from easily accessible wild populations; and that they represent diverse phyla and that, in the case of the animal species, represent different levels in the food web (Tables 1 and 2).



Figures 1 and 2. *Schematics of the multispecies test systems that was used to test mod MPCAs with marine (Figure 1) and freshwater (Figure 2) non-arg species.*

Mass Culture of NTO Test Species

Culture and holding facilities for laboratory colonies of the marine and freshwater NTO species consisted of four totally self contained, recirculating multiple tank systems (of approximately 1500 L each in volume). These were located in a sheet metal building and a plastic covered greenhouse on the grounds of the University of Arizona's Environmental Research Laboratory (ERL). Two systems (one each in the greenhouse and metal building) were used for the mass culture of marine NTOs, while the remaining two systems were used to mass culture the freshwater NTOs.

Feeds and Feeding Methods for NTOs

Marine and freshwater NTOs in the large rearing tanks and in the MPCA test tanks were fed live food organisms or frozen and artificial feeds once each day that were consistent with their feeding behavior and known nutritional requi ments. Thus, filter feeding mollus were fed cultures of planktonic alg once per day. Finfish and grass shri received chopped frozen squid, arter nauplii, and a commercial flake fo daily. NTO species that were not directly included the aquatic pla (*Salicornia* and *Elodea*), the snails, tubifex worms, and the shore fly larvae

Model MPCAs Tested

Four model MPCAs were tested w marine and freshwater NTOs in our t

Table 1. Assessment of Marine Non-Target Species Used in Tests with Model MPCAs.

Marine Species	Source[1]	Lab Culture[2]	Test Organism Suitability
Plant			
Salicornia bigelovii *(intertidal halophyte)*	*Gulf of CA*	*RP*	*Poor*
Invertebrate Animals			
Bunodosoma californica *(sea anemone)*	*Gulf of CA*	*RP*	*Fair*
Turbo fluctuosus *(turbin snail)*	*Gulf of CA*	*CW*	*Excellent*
Crassostrea gigas *(Japanese oyster)*	*Gulf of CA*	*PS*	*Excellent*
Palaemonetes pugio *(estuarine grass shrimp)*	*Florida*	*RP*	*Excellent*
Ephydra sp. *(shore fly)*	*Gulf of CA*	*RP*	*Fair*
Vertebrate Animal			
Cyprinodon variegatus *(sheepshead minnow)*	*Florida*	*RP*	*Good*

[1]*Source. Gulf of CA = collection sites near Puerto Penasco in Sonora, Mexico, on the Northern Gulf of Mexico.*
[2]*Lab culture: RP = reproducing laboratory colony established.*
CW = captive wild colony successfully maintained in lab
PS = experimental animals purchased from a commercial supplier and maintained in lab.

system. Used in these studies were three bacterial MPCAs (a spore forming *Bacillus*, a vegetative form of Bacillus, and a *Pseudomonas*), and an insect baculovirus (Table 3).

Spore-Forming Bacillus sphaericus

Spores of *Bacillus sphaericus* (modified strain 2362) containing the plasmid pLT103 were used in Trials 1 and 5. This strain of *B. sphaericus* possesses insecticidal activity against mosquitoes and it is being developed commercially for use as a mosquito larvacide. In addition to the strain's natural resistance to streptomycin, insertion of the plasmid, pLT103, that encodes for Neomycin resistance, provided the bacterium with a unique genetic marker, which was used to "track" this organism in the MPCA tests using conventional microbiological methods.

Vegetative Cells of Bacillus sphaericus

Vegetative cells of *B. sphaericus* (modified strain 1593, thymine deficient and harboring the plasmid pLT117) were used in Trial 2 The plasmid (pLT117), a ligation product of pTG402 and pUB110, encodes for Neomycin resistance, as well as containing the xylE gene. The xylE gene expresses catechol 2,3-dioxygenase which converts catechol from colorless to a yellow product (2-hydroxymuconic semialdehyde) within a few minutes when sprayed onto growing colonies. Culture and detection methods for this organism consisted of supplementing TBAB agar plates with Neomycin (5 µg/ml) and thymine (50 µg/ml). After overnight incubation, plates were sprayed with catechol solution and observed for yellow colonies

Pseudomonas putida *with Genetic Markers*

In Trials 4 and 6 vegetative cells of a genetically altered strain of *Pseudomonas putida* were used. While this species has no uses as an MPCA, it was selected for use here as a model for other members of the genus that are being developed as MPCAs. This strain had been modified from the parent strain PPO200, by transformation with the plasmid pEPA74 This plasmid was constructed by inserting the UC19 multiple linker sequence and a piece of plant DNA (approximately 400bp) into a pseudomonas plasmid pKT230 which contains Kanamycin resistance. The resulting pseudomonad was mutated in two separate genes on the chromosome to produce a strain resistant to high levels of nalidixic acid. By using Pseudomonas F Agar, (a selective medium for Pseudomonas spp. and upon which colonies are fluorescent yellow), supplemented with 500 µg/ml nalidixic acid and 150 µg/ml Kanamycin, we were able to select exclusively for the genetically altered strain of *P. putida.*

A gene probe was used, in addition to the traditional microbiological methods, to "track" this model MPCA in the test system The gene probe was prepared by insertion of the 400 bp segment of plant DNA into a Pst1/EcoR1 site on a pUC18 plasmid and then transformed into *E. coli* Ac80. This organism provided plasmid DNA for labeling, which was used as a gene probe.

The Nuclear Polyhedrosis Virus AcMNPV

The nuclear polyhedrosis baculovirus (AcMNPV) from the lepidopteran *Autographa californica* was used as the model MPCA in Trials 3 and 7. The JM83 strain of E. coli that harbors a pUC18 plasmid, which contains a pAC HindV insert (1000 bp) of the central region of the polyhedrin gene of AcMNPV, was used as a gene probe to "track" this model MPCA in the test system.

Containment of MPCAS in the Test Systems

The building in which tests with wild-type and genetically altered model MPCAs were conducted was located at the extreme eastern end of the ERL grounds. It was isolated from other occupied buildings at the facility by at least 30 meters. The building was constructed to provide a limited access "containment" area. A wall separated the experimental half of the building, where MPCAs were tested with NTOs in glass aquaria, from the entry and NTO mass culture tanks areas of the building. To further insure containment of the model MPCAs, rubber boots (disinfected in a 200 ppm chlorine foot bath at the entrance door) were required for access to the experimental side of the building. In addition, nets, labware, and other tools used in the test tanks for sampling purposes were labeled and dedicated to a particular tank (in order to reduce

Table 2. *Assessment of Freshwater Non-Target Species Used in Tests with Model MPCAs.*

Freshwater Species	Source	Lab Culture[1]	Test Organism Suitability
Plant			
Elodea canadensis *(Anacharis plant)*	*Commercial*	*S*	*Excellent*
Invertebrate Animals			
Tubifex tubifex *(annelid worm)*	*Commercial*	*PS*	*Fair*
Gyraulus sp. *(snail)*	*Arizona pond*	*RP*	*Good*
Margaritifera margaritifera *(freshwater mussel)*	*Commercial*	*PS*	*Excellent*
Palaemonetes kadiakensis *(freshwater grass shrimp)*	*Commercial*	*RP/PS*	*Good*
Vertebrate Animal			
Poecilia latipinna *(sailfin molly)*	*Hawaii*	*RP*	*Excellent*

[1]*Lab culture:*
RP = reproducing laboratory colony established.
PS = experimental animals purchased from a commercial supplier and maintained in the laboratory.

Table 3. *Model MPCAs and dose rates applied to multispecies aquatic test systems in Trials 1 through 7**

Trial Number	Model MPCA	Initial Dose Level
1 (M)	Bacillus sphaericus *spores*	*10^6 CFU/ml*
2 (M)	B. sphaericus *vegetative cells*	*2 x 10^6 CFU/ml*
3 (M)	*AcMNPV baculovirus occlusions*	*10^6 occlusion bodies/ml*
4 (M)	Pseudomonas putida	*10^6 CFU/ml*
5 (M)	*B. sphaericus spores*	*1.6 x 10^7 CFU/ml*
6 (FW)	Pseudomonas putida	*7.5 x 10^4 CFU/ml*
7 (FW)	*AcMNPV baculovirus occlusions*	*1.8 x 10^5 occlusion bodies/ml*

**M = test run in saltwater with marine NTO species.*
FW = test run in freshwater with FW NTOs.
CFU = colony forming units.

cross-contamination) and disinfect separately in 100 ppm iodine (polyvi providine iodine; Fritz Egg Disinfecta Fritz Chem. Co., Dallas, TX). The fl was mopped with 100 ppm pvp iod periodically to further reduce the risk contamination. A 5000 liter concre sump received waste water from MPCA trials. Water contained in t sump was continuously chlorinated (to 20 ppm chlorine) prior to disposal.

General Methods for MPCA Tests

In all tests with model MPCAs, six s contained 120 L glass aquaria (three t and three control) were used. T biological filters in each aquarium we pre-conditioned with a commerc preparation of nitrifying bacteria (Aq Gold, LaMonte Environmental Techn ogy, Saticoy, CA) or by addition of fil matrix material from "mature" functic ing filters. Artificial seawater (Fo Fathoms, Marine Enterprises, Towso MD) was used in Trials 1 through 5; c tap water was used to make up t artificial seawater. City tap water w used directly in the freshwater Trials and 7. Salinity, pH, ammonia, nitri dissolved oxygen, and alkalinity of t tank water were monitored and ma tained (by aeration, partial wat exchanges, manipulation of feeding ra use of room space heaters/coolers, et within acceptable limits.

Each of three replicate test and cont tanks were stocked with 15 NTOs each species. This provided a total of NTOs of each species in test and cont treatments of each MPCA test. Th number was sufficient to provide microbiological and histologic sampling, as well as to allow for sor loss due to possible natural and/ treatment related mortalities, while s providing for statistical confidence data evaluation.

Dosing and Sampling Methods

Three test tanks were inoculated day 0 of a planned 28 to 30 day stu with the model MPCAs to dose leve listed in Table 3. The amount of mod MPCA added to each test system w representative of amounts found in t literature or recommended by the ma ufacturers of our model (or simila MPCAs for use in field applications insect control. The water was then mix for 5 min, and water samples were tak from each tank to determine the init concentration of recoverable mod MPCA. At predetermined time interva

Table 4. *Summary of Microbiological Assays for* Bacillus sphaericus *Spores in Trial 5 Run in Seawater*

	Controls										Exposed									
	NTOs						Tank Water				NTOs						Tank Water			
Day of Trial	F	GS	O	S	P	A	9	10	11		F	GS	O	S	P	A	1	2	3	
0		0		0	0	0	0	0	0	0		0		0	0	0	0	0	0	0
i	----------nd----------										----------nd----------									
1	0	0	0	0	0	0	0	0	0		+	+	+	+	+	+	+	+	+	
5	----------nd----------						0	0	0		+	+	+	+	+	+	+	+	+	
12	0	0	0	0	0	nd	0	0	0		+	+	+	+	+	+	+	+	+	
15	0	0	0	0	0	0	0	0	0		+	+	+	+	+	nd	+	+	+	
30	0	0	0	0	0	nd	0	0	0		+	+	+	+	+	+	+	+	+	

Abbreviations used:
0 = MPCA not recovered from the test system water or NTOs.
+ = MPCA recovered from test system water or NTOs.
i = sample taken immediately after MPCA introduced.
F = sheepshead minnow (fish)
GS = grass shrimp
S = turbin snail
O = oyster
P = Salicornia sp. (plant)
A = sea anemone
nd = not done

throughout the study, water and NTO samples were collected for microbiological and histological analyses.

Water: Sterile pipets were used to collect approximately 5 ml of water from each control and test tank. The water was placed into sterile plastic tubes and kept on ice until assayed. For microbiology assays, 0.1 ml was dropped into the middle of the appropriate media plate, spread with a sterile "hockey stick" (a bent glass rod) and placed in an incubator (30°C for *P. putida* and 37°C for *B. sphaericus*). For the *B. sphaericus* vegetative cell study, the plates were sprayed with catechol following overnight incubation. For gene probe assays, the water was stored at -20°C until the gene probe assay was performed.

Tissues: NTOs were placed in plastic bags on ice once removed from the experimental tanks. At the lab, the organisms were surface sterilized by soaking them in Fritz's egg disinfectant for 5-10 min. Oysters, mussels, snails and fish were scrubbed with a brush before put into two washes of sterile distilled water. The remaining organisms were washed two times with sterile. distilled water. For analysis tissue samples were aseptically removed and homogenized in 0.01 M Tris buffer at pH 7.0. One hundred microliters of each resulting homogenate were plated and spread onto duplicate plates of the appropriate media and incubated. The remainder of the sample was stored at -20°C for subsequent gene probe assays. For the gene probe assay, 0.5 ml of each sample was added to 0.5 ml deionized formamide and incubated for 30 min at 80°C to liberate nucleic acid. The samples were then applied to a Gene Screen plus hybridization membrane, baked at 80°C for 2 hr, prehybridized, hybridized, washed and placed on X-ray film to produce an autoradiogram.

Histological Samples: Samples for histological examination were preserved in Davidson's AFA fixative for 24 to 76 hr, transferred to 50% ethanol for storage, and later processed and examined using routine histological methods. Mayer's hematoxylin and phloxine/eosin stain was used for all NTO specimens. In addition, Brown and Brenn tissue Gram stain was used in those trials in which bacterial model MPCAs were used.

Method for Obtaining Plasmid DNA for Gene Probes

For the studies using the MPCAs AcMNPV and *P. putida* (Table 3), plasmid DNA was radio-labeled and used as gene probes. Both plasmids originated from the pUC 18 family of plasmids and were placed in *E. coli* strains, and both plasmids contained an Ampicillin resistance gene. To harvest large amounts of the DNA, the appropriate *E. coli* was grown in LB media supplemented with 30-50 µg/ml Ampicillin. Overnight cultures were pelleted, the bacteria washed and the plasmid was isolated using the alkaline lysis procedure.

Results and Discussion

Test Systems

An enclosed test system was developed in which multiple species of aquatic animals and plants were tested for adverse non-target effects following experimental exposure to wild-type or genetically altered MPCAs. The test system was used to test several model MPCAs representative of those being developed for possible registration and use in the United States.

Non-Target Species

A number of marine, estuarine, and freshwater animal and plant species were collected and evaluated for possible use as non-target species in multispecies test systems with model MPCAs. Some species selected as NTOs proved to be excellent experimental species in terms of their availability, ease of laboratory culture, and representation of important

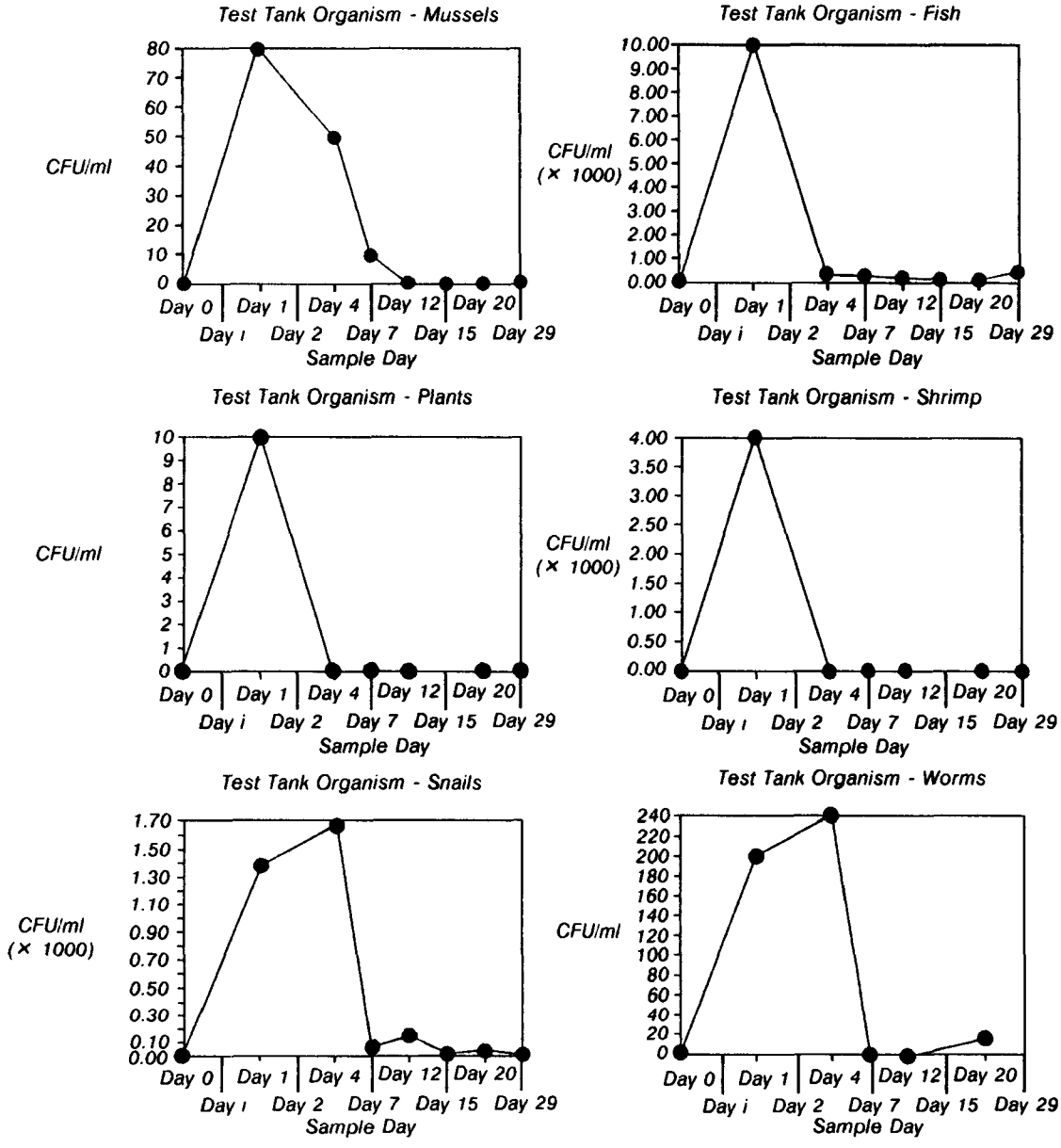


Figure 3. *Graphs representing microbiological results for detection of* Pseudomonas putida *in non-target organisms from test tanks in Trial 6. No* P. putida *was detected in control samples.*

phylogenetic groups in aquatic ecosystems (Tables 1 and 2).

While no adverse effects were noted in any NTO as a result of exposure to model MPCAs (i.e., in terms of survival, gross appearance and histology of control and exposed specimens), data from the sea anemone, saltwater plant, and shore fly larvae were difficult to interpret due to problems with their use in the enclosed aquaria. The sea anemones moved between sampling times, were difficult to find, and, therefore, were not sampled during each scheduled sampling period. In addition, nematocysts filaments of the sea anemone stained Gram positive and fragments of these in histological sections were so similar in size to *Bacillus sphaericus* vegetative rods as to be difficult to distinguish. The saltwater plant *Salicornia* that was used in Trials 1-5 frequently browned and wilted. The shore fly larvae were difficult to study as they pupated and adults emerged usually well before the end of a 28 day trial.

Of the freshwater species liste
Table 2, only the tubifex worms
sented problems in their use as N
by not surviving well. Surface foulir
the worms by a filamentous blue g
probably *Schizothrix calcicola* and
tain diatom species, was considere
be the cause of the poor survival of t
worms. In nature tubifex worms
embedded in bottom sediments (not
vided in the test system described h
which protect them from light and su
fouling organisms. If tubifex worms

be protected from predation in a multi-species test system, while being provided with a substrate in which to burrow, they might otherwise make an excellent NTO species.

MPCAs Tests and Detection Methods

The model MPCAs utilized in these studies provided a range of fates and persistences in the enclosed multi-species test system. In the two Trials (1 and 5) in which *B. sphaericus* spores were used as the model MPCA, the organism persisted in saltwater throughout the 28 day duration of the two studies. This was anticipated because B. sphaericus spores are known to remain viable in soil for considerable periods of time and to remain visibly unaffected during passage through the gut of mosquitoes.

Spore-Forming Bacillus sphaericus

The detection method used to track *B. sphaericus* in Trials 1 and 5 was simple and easy to use. The presence of the MPCA was readily determined, and it was accurately enumerated. NTO histological studies in these Trials showed the presence of abundant Gram positive bacilli in the gut contents of some of the NTOs from the exposed tanks. This observation suggests that the model MPCA may have cycled through the food chain. However, although this model MPCA did persist for at least 30 days in the test system, while losing three logs activity, it did not cause observable pathological anomalies in the NTOs (Table 4).

Vegetative Cells of Bacillus sphaericus

In marked contrast to the findings when bacillus spores were used as the model MPCA, the vegetative cells of the strain of *B. sphaericus* used in Trial 2 became undetectable in the seawater system within 24 hr. Histological study of the NTOs in this trial also suggested that the NTOs consumed the MPCA, but that its presence caused no pathological anomalies.

Our inability in Trial 2 to recover viable *B. sphaericus* vegetative cells after 24 hr from our test system may suggest that the bacterial cells were destroyed by environmental effects and possibly by the NTOs. This latter route of MPCA clearance from test tanks is a possibility because one large oyster may filter nearly 400 L of seawater in 24 hr. As each 120 L aquarium contained 15 oysters at the start of each trial, the entire volume of tank water may have passed through the oysters as many as 50 times in the first 24 hr. If only a fraction of the viable *B. sphaericus* cells were inactivated during each passage through the gut of an oyster, it is possible that the entire dose of MPCA could be reduced to zero in a single day.

Pseudomonas putida *with Genetic Markers*

Pseudomonas putida used as a model MPCA in Trials 4 and 6 showed variable results. In the saltwater test system, it did not survive more than 5 days, but in the freshwater system it did survive and it was detectable in some samples for the duration of the 29 day study (Figure 3). Interestingly, the results showed that the tubifex worms, freshwater snails, and the mollies harbored the MPCA, although in just detectable amounts, for the duration of the Trial. In contrast, the MPCA was not detectable in the test tanks' water by day 4. These findings suggest that *P. putida* had colonized certain of the NTOs, becoming part of their microflora. In both marine and freshwater trials with this MPCA, gross signs, survival, and histological study of control and MPCA exposed NTOs showed no differences and no adverse effects attributable to the MPCA.

The microbiological culturing method was excellent for tracking *P. putida* in the test system and in the tissues of the NTOs. The combination of the two antibiotic resistance genes, in addition to the biochemical properties inherent in this *Pseudomonas* sp. (i e , turning Pseudo F agar yellow under its colonies), simplified isolation, identification, and enumeration of this genetically engineered microorganism. The gene probe assay for tracking this organism (*Pseudomonas*) in saltwater (Trial 4) also worked very well However, in the freshwater system (Trial 6), the assay was not sufficiently specific, as there was some non-specific binding or cross reaction with the probe. Possibly, this was due to the presence in the freshwater system of one or more other *Pseudomonas* sp. If a gene probe method for this organism is to be used in the future in a freshwater system, it will be necessary to do more investigations with the gene probe and determine the extent of the interference.

Nuclear Polyhedrosis Virus AcMNPV

In Trials 3 and 7 a gene probe to the polyhedrin gene of the baculovirus AcMNPV was employed to detect and track the fate and persistence of the viral DNA of this model MPCA. The results of these Trials indicated that the virus could be detected in the test tank water using the gene probe on the initial day of the seeding of the water, but not on subsequent days of the study in test system water or associated with the NTOs. be detected in the test tank water using the gene probe on the initial day of the seeding of the water, but not on subsequent days of the study in test system water or associated with the NTOs.

Therefore, either the baculovirus did not persist in the test tanks' seawater or in the tissues of the NTOs beyond day 1 of the two trials, or the probe lacked adequate affinity to the viral DNA to demonstrate its presence. While AcMNPV occlusion bodies were observed in the gut contents of snails sampled 24 hr after exposure in Trial 7, gross signs, survival, and histological study of control and MPCA exposed NTOs showed no differences and no adverse effects attributable to the MPCA.

Conclusions and Recommendations

Further studies with model MPCAs in multispecies aquatic test systems should include analysis of test system water and NTO tissue homogenates for specific antigens or nucleic acid from the model MPCAs using either monoclonal antibodies or gene probes. It is important to know the fate or persistence of not only the intact viable model MPCA itself, but the fate of its genetic material as well. Furthermore, the use of the recently developed polymerase chain reaction to amplify the genetic material may enhance sensitivity of MPCA detection when using gene probes. Because the microorganisms present in the gut contents of several NTO species in the present study may have been the model MPCAs used, future studies that positively identify these organisms "in situ" using specific antibodies or gene probes are recommended.

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The complete report, entitled "An Enclosed Aquatic Multispecies Test System for Testing Microbial Pest Control Agents with Non-Target Species," (Order No. PB 89-231 526/AS; Cost: $15.95, subject to change) will be available only from:
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