Environmental Risk Assessment for Toxic Chemicals

and Genetically-Engineered Microorganisms:

A Microcosm Approach

by

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Dissertation submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Biology

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April, 1990

Blacksburg, Virginia

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(ABSTRACT)

Microcosms were used in this research to assess the environmental risk associated with two types of stressors: genetically-engineered microorganisms (GEMs) and toxic chemicals. Approaches used to evaluate the potential environmental impact of these two stressors are fundamentally different, and arise mainly from the fact that the GEMs are living and capable of replication. Risk assessment for genetically-engineered *Erwinia carotovora* strain L-864 included investigations of its persistence and effect on structure and function of aquatic microcosms. Densities of genetically-engineered and wildtype *E. carotovora* declined at the same rate in water or in sediment, falling in 32 days below the level of detection by viable counts. Selective media, antibiotic resistance, and most probable number analysis were used to enumerate genetically-engineered *E. carotovora* in environmental samples. This technique was able to detect as few as 1 to 10 target cells/10 g soil. In thermally-perturbed aquatic microcosms, genetically-engineered *E. carotovora* persisted significantly longer than in unperturbed microcosms, suggesting the vulnerability of stressed ecosystems to colonization by GEMs. Competition study showed that the genetically-engineered *E. carotovora* did not displace the wildtype strain. Effects of genetically-engineered and wildtype strain on indigenous bacteria belonging to specific functional groups important in nutrient cycling were similar: inoculation of either strain caused a temporary increase in densities of total and proteolytic bacteria,

while it did not affect amylolytic and pectolytic bacteria. Treatment with engineered bacteria did not change biomass values of the receiving community, but caused a transitory increase in its metabolic activity. The inability of genetically-engineered *E. carotovora* to persist, displace resident species, and affect metabolic activity of a community indicates a low risk of adverse ecological effects in aquatic systems. Microcosms were also used to assesss environmental risk for toxic chemicals. A study was conducted to assess the fate of sediment-associated copper and to investigate its effects on the structure and function of the aquatic community. Most of the added copper was bound to sediment particles. In microcosms containing 100 μg Cu/kg sediment, chlorophyll *a* content and respiration significantly decreased compared to the control. Addition of 1000 μg Cu/kg sediment caused a decrease in production, respiration, respiration/biomass ratio, ATP, and chlorophyll *a*. The last study compared responses of *Simocephalus exspinosus* (Daphnidae) to copper during a single-species test to responses of *S. exspinosus* populations in a microcosm test. Responses of *S. exspinosus* were similar in both test systems: there was an increased production of young at 30-46 μg Cu/l, while the organisms did not survive exposure to concentrations > 100 μg Cu/l. In these studies, microcosms showed a potential to predict fate and effects of chemical and biological contaminants released into the biosphere.

Acknowledgments

This research was supported in part by E. I. du Pont de Nemours Educational Foundation, the Mobil Foundation Incorporated, Sigma Xi Grant-in-Aid of Research, grant BSR-8705445 from Natural Science Foundation, grant 813805-01-0 from the Environmental Protection Agency, Graduate Student Assembly Graduate Research Development Project, and Graduate Student Assembly Travel Fund Program. Department of Biology of Virginia Tech provided matching funds for the grants, tuition wavers, and teaching assistantships.

I am deeply indebted to my adviser Dr. John Cairns, Jr. for his scientific guidance, encouragement, and necessary financial support during the various stages of this research. I am grateful to my committee members, Dr. George Lacy, Dr. Robert Paterson, Dr. Eric Smith, and Dr. Bruce Wallace for their helpful suggestions, comments on manuscripts, and critical reviews of the dissertation. I owe a special thanks to Dave Orvos and Bobbie Niederlehner for their advice and stimulating discussions, to Darla Donald for preparing manuscripts for publication, and to Julie Jim for excellent technical assistance. My appreciation is also extended to Rob Atkinson, Jay Comeaux, Will

Clements, Paul McCormick, and Kurt Pontash. Lastly, I would like to thank my husband Jose Luis Scanferlato for his support, help, and understanding.

Table of Contents

Prologue

This research deals with environmental risk assessment associated with two types of stressors: toxic chemicals and genetically-engineered microorganisms (GEMs). In order to predict the changes that might result from the presence of these stressors in the environment, various risk assessment methods have been developed. The risk associated with chemical substances released into the environment has been traditionally evaluated using laboratory bioassays. The efficacy and suitability of such standardized test methods for predicting community-level responses to toxicants is currently being questioned (Cairns, 1989; Kimball and Levin, 1985; Moore and Winner, 1989). Risk assessment methods for genetically-engineered microorganisms intended for environmental use are still under development (Dean-Ross, 1986; Fiksel and Covello, 1986; Tiedje et al., 1989).

In the studies reported here, microcosms were used to assess the environmental risk associated with genetically-engineered microorganisms and toxic chemicals. Microcosm systems were used as surrogates of natural ecosystems because of their potential to provide information on the fate and effects of chemical and biological contaminants released into the biosphere.

Microcosm has several definitions. According to Giesy and Odum (1980), microcosms are "artificially bound subsets of naturally occurring environments which are replicable." Leffler (1980) defines them as "small, living models of ecosystem processes," and Suter (1985) as "laboratory systems designed to physically simulate some portion of the ambient environment." Microcosms are used because they bridge the gap between laboratory tests and field tests. They can be employed to predict deleterious effects of physical, chemical, or biological stressors on ecosystems.

Both GEMs and toxic chemicals are similar in that they can cause perturbations in ecosystem structure and function when released into the environment; however, approaches used to evaluate their potential environmental impact are fundamentally different. Investigation of potential deleterious effects due to GEMs presence requires knowledge of a microorganism's genotype and its biological and ecological properties. The problems that complicate risk assessment arise mainly from the fact that the stressor is living. The GEMs may exchange their engineered DNA with the other microorganisms, and, although not harmful in the original GEM, the manipulated DNA may become expressed and act unpredictably in the new host (Comeaux, 1989). The transport of GEMs to habitats away from the site of their release and the possibility of their persistance in undetectably low concentrations are important in assessing the risk in GEMs release (McCormick, 1988). Since GEMs, unlike toxic chemicals, may multiply and increase in concentration under favorable conditions, the dose-effect paradigm cannot be applied (Cairns and Pratt, 1986).

In order to determine the risk associated with genetically-engineered *Erwinia carotovora*, this study used microcosms that simulate the environment of interest. Microcosms were contained (to minimize risk of GEMs escape) and small in size (to ensure intact decontamination). This approach eliminated the dangers associated with field tests that result from the absence of reliable recall mechanisms for GEMs.

Risk assessment of genetically-engineered *E. carotovora* included investigation of its persistence and its effect on microcosm structure and function. Colonization by GEMs is most likely to succeed in ecosystems from which the predators and competitors have been eliminated; therefore, this study investigated the survival of genetically-engineered *E. carotovora* in a system in which the chance for colonization was maximized (Chapter 3). Since the ability to detect GEMs present in low densities in environmental samples is important in evaluating any risk in their release, an inexpensive, quantitative, and sensitive enumeration technique was developed (Chapter 2). The persistence of genetically-engineered *E. carotovora* in the presence of its wildtype parent in a competition study was also investigated (Chapter 4). The impact of genetically-engineered and the wildtype strain on indigenous bacteria belonging to specific functional groups important in nutrient cycling is described in Chapter 1. Chapter 4 describes the effects of GEMs on structural groups of indigenous bacteria, on biomass, and on the metabolic activity of the receiving community.

The potential effects of chemical substances released into the environment have been determined traditionally by laboratory toxicity bioassays. Such an approach has been often criticized for lack of environmental realism and its inability to accurately predict the response of the ecosystem receiving the toxic materials (Cairns, 1989; Giesy and Odum, 1980; Kimball and Levin, 1985). Recently, scientists and regulatory agencies became increasingly aware of the potential of microcosms in studying the fate and effects of chemical contaminants released into the biosphere. Microcosms possess a greater environmental complexity than single-species tests, integrate responses of higher levels of biological organization to toxic stress, and enable impact studies on system-level properties that cannot be done at the lower levels of biological organization (Kimball and Levin, 1985). The advantages of microcosm over ecosystem studies are replicability, that enables statistical testing of the effects of manipulation; reduced residual variability

typical of natural systems; and lack of environmental damage that might occur in exposing natural systems (Giesy and Odum, 1980). Because of these properties, microcosm tests are considered to be effective tools in environmental impact assessment.

Microcosms were used to assess the fate of sediment-associated copper and to investigate its effects on the structure and function of the aquatic community (Chapter 5). The study described in Chapter 6 compared responses of individuals to a toxic substance during a single-species test to the responses of populations of the same species in a more complex system.

Some caution should be applied in interpreting results of microcosm tests. Natural ecosystems are far more complex than microcosms, and they should never be treated as a small replica of a real-world situation (Harte et al., 1980; King, 1980). Because of their isolated nature, microcosms do not have a normal influx of materials; diurnal and seasonal changes that occur in nature are difficult to simulate in microcosms; and they suffer from the "container effect" since they have an unrealistic surface to volume ratio. The behavior of toxic substances or genetically-engineered microorganisms may be altered in microcosms, and, thus, the extrapolation of results to the specific ecosystem should be performed with caution. Microcosms are useful as screening tools for preliminary evaluation of ecological fate and effects, but the results require validation on a larger scale. Hazard assessment should be performed across the levels of ecological complexity, starting with simple, laboratory tests and moving toward the tests that are more complex and environmentally realistic (Cairns, 1989; Dean-Ross, 1986).

LITERATURE

Cairns, J., Jr. 1989. Applied ecotoxicology and methodology, p. 275-290. *In:* A. Boudou and F. Ribeyre (eds.), Aquatic Exotoxicology: Fundamental Concepts and Methodologies. CRC Press, Inc., Boca Raton, Fla.

Cairns, J., Jr. and J. R. Pratt. 1986. Ecological consequence assessment: effects of bioengineered microorganisms. Water Res. Bull. 22:171-182.

Comeaux, J. L. 1989. Transfer of plasmids by genetically-engineered microorganisms. M.S. Thesis. Virginia Polytechnic Institute and State University, Blacksburg.

Dean-Ross, D. 1986. Applicability of chemical risk assessment methodologies to risk assessment for genetically engineered microorganisms. RAC Tech. Bull. 9:16-28.

Fiksel, J. R. and V. T. Covello. 1986. The suitability and applicability of risk assessment methods for environmental applications of biotechnology, p. 1-34. *In:* J. Fiksel and V. T. Covello (eds.), Biotechnology Risk Assessment: Issues and Methods for Environmental Introductions. Pergamon Press, New York.

Giesy, J. P. and E. P. Odum. 1980. Microcosmology: introductory coments, p. 11-13. *In:* J. P. Giesy (ed.), Microcosms in Ecological Research. Technical Information Center, U.S. Department of Energy, Springfield, Va.

Harte, J., D. Levy, J. Rees, and E. Saegebarth. 1980. Making microcosms an effective assessment tool, p. 105-137. *In:* J. Fiksel and V. T. Covello (eds.), Biotechnology Risk Assessment: Issues and Methods for Environmental Introductions. Pergamon Press, New York.

Kimball, K. D. and S. A. Levin. 1985. Limitations of laboratory bioassays: the need for ecosystem-level testing. BioScience 35:165-171.

King, D. L. 1980. Some cautions in applying results from aquatic microcosms, p. 164-191. *In:* J. Fiksel and V. T. Covello (eds.), Biotechnology Risk Assessment: Issues and Methods for Environmental Introductions. Pergamon Press, New York.

Leffler, J. W. 1980. Microcosmology: theoretical applications of biological models, p. 14-29. *In:* John P. Giesy (ed.), Microcosms in Ecological Research. Technical Information Center, U.S. Department of Energy, Springfield, Va.

McCormick, D. 1988. Detection technology: The key to environmental microbiology. Bio/Technology 4:419-422.

Moore, M. V. and R. W. Winner. 1989. Relative sensitivity of *Ceriodaphnia dubia* laboratory tests and pond communities of zooplankton and benthos to chronic copper stress. Aquat. Toxicol. 15:311-330.

Suter, G. W. 1985. Application of environmantal risk analysis to engineered organisms, p. 211-219. *In:* H. O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environmant: Scientific Issues. American Society for Microbiology, Washington, D.C.

Tiedje, J.M., R. K. Colwell, Y. L. Grossman, R. E. Hodson, R. E. Lenski, R. N. Mack, and P. J. Regal. 1989. The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. Ecology 70:298-315.

Chapter 1

Genetically-Engineered *Erwinia carotovora* in Aquatic Microcosms: Survival and Effects on Functional Groups of Indigenous Bacteria *

ABSTRACT

Survival of genetically-engineered *Erwinia carotovora* strain L-864, with a kanamycin resistance gene inserted in its chromosome, was followed in the water and sediment of aquatic microcosms. The density of genetically-engineered and wildtype *E. carotovora* strains declined at the same rate, falling in 32 days below the level of detection by viable

* This chapter is published in Appl. Environ. Microbiol. 55:1477-1482, with the authors V. S. Scanferlato, D. R. Orvos, J. Cairns, Jr., and G. H. Lacy.

counts. We examined the impact of the addition of genetically-engineered and wildtype strains on indigenous bacteria belonging to specific functional groups important in nutrient cycling. Up to 16 days, the densities of total and proteolytic bacteria were significantly higher ($p < 0.05$) in microcosms inoculated with genetically-engineered or wildtype *E. carotovora*, but decreased 32 days after inoculation to densities similar to those in control microcosms. Inoculation of genetically-engineered or wildtype *E. carotovora* had no apparent effect on the density of amylolytic and pectolytic bacteria in water and sediment. Genetically-engineered and wildtype *E. carotovora* did not have significantly different effects on the densities of specific functional groups of indigenous bacteria ($p > 0.05$).

INTRODUCTION

The risk related to the introduction of genetically-engineered microorganisms (GEMs) depends on their establishment in the environment. Only if the GEMs have the ability to survive, multiply, or transfer genetic material to other organisms within an ecosystem will they have a lasting impact and cause significant perturbations (28). Therefore, ability to predict the survival and colonization of GEMs within an ecosystem is a key element in assessing risk of their release (29). Because GEMs may spread to adjacent ecosystems, it is also essential to determine their survival under conditions that differ from those within the system into which they are released (5).

GEMs introduced into the environment interact with indigenous populations (16, 17, 26). There is concern that GEMs may displace indigenous bacterial populations important in nutrient cycling and detritus mineralization in aquatic systems. Thus, GEM introduction might alter fundamental processes, such as energy, carbon, and nutrient cycling, that rely mainly on bacterial species or products (5, 9). This is especially important in aquatic ecosystems in which the greatest amount of biomass and residual carbon is in the form of detritus and its processing accounts for the greatest amount of

energy (31). Bacteria important in decomposition and mineralization are considered to be susceptible to the effects of GEMs (5, 6).

Bacteria belonging to definite functional groups can indicate occurrence of certain ecological processes in the ecosystem, such as transformations of various compounds of carbon, nitrogen, and phosphorus (22). Amylolytic, proteolytic, and pectolytic bacteria are among the groups involved in detritus mineralization and their presence is characteristic of a certain type of degradation in the system. Bacteria from each functional group can be isolated from its natural habitat and detected by growth on selective media (10).

A genetically-engineered strain of the plant pathogen *Erwinia carotovora* was chosen for this study for its potential ecological and economic significance as a biological control agent. Persistence and effects due to the introduction of genetically-engineered *E. carotovora* have not yet been examined in an aquatic environment. Erwiniae seem to be commonly present in aquatic systems (11, 13, 18). Certainly, water is important in the transport of these pathogens (3, 15). The objectives of the study were: (a) to assess the persistence of the genetically-engineered and wildtype *E. carotovora* in aquatic microcosms, and (b) to determine the potential impact of genetically-engineered and wildtype *E. carotovora* on certain functional groups of indigenous bacteria involved in mineralization.

MATERIALS AND METHODS

Bacterial strains. *Erwinia carotovora* subsp. *carotovora* is a plant pathogen that causes soft rot and produces a battery of enzymes (pectate lyases, cellulases, phospholipases, and proteases) that are responsible for the breakdown of plant tissue components (7, 14). The genetically-engineered *E. carotovora* strain L-833 was constructed from wildtype *E. carotovora* strain L-543 by disarming *in vitro* an extracellular

pectate lyase on a plasmid by deletion mutation, inserting a DNA fragment from Tn*903* conferring kanamycin resistance, and chromosomally inserting part of the engineered plasmid in a *cis* relationship to the wildtype pectate lyase gene in the pathogen's chromosome (2). Genetically-engineered strain L-833 is 30% reduced in its ability to rot potato tuber tissue compared to the wildtype (Fig. 1). We used spontaneous rifampicin mutants of L-543 and L-833 designated L-863 and L-864, respectively, in these studies to aid in isolation. These mutants are resistant to 150 ug/ml rifampicin. Growth and biochemical characteristics of the genetically-engineered strain are identical to the wildtype strain (data not shown).

Microcosm preparation and sampling. Simple microcosms were constructed from 850 ml glass Mason jars. Sterile jars were filled with 300 ml of sediment collected from Pandapas Pond, Montgomery County, Virginia, which has been described elsewhere (4), and covered with 550 ml of water from the same site. A flow-through system delivered air to the water column. Microcosms inoculated with different doses of genetically- engineered or wildtype *E. carotovora* and uninoculated control microcosms were incubated at 20°C and illuminated on a 12-h light 12-h dark cycle.

Preparation of bacterial cultures, inoculation, and sampling. Cultures of genetically-engineered and wildtype *E. carotovora* were grown for 24 h in nutrient broth at 30° C, with shaking at 150 rpm, to a density of approximately 10^9 CFU/ml estimated turbidiometrically at 550 nm. Cells were harvested by centrifugation at 10,000 x *g* for 15 min, washed in sterile distilled water, recentrifuged, and resuspended in 10 ml of sterile water. Microcosms were inoculated into the water phase with 10^3, 10^6, or 10^9 CFU/ml to assess the survival of *E. carotovora* in aquatic microcosms. The bubbling aerator mixed the inoculum into the water. The effect of *E. carotovora* on indigenous aquatic bacteria was assessed in microcosms inoculated with 10^9 CFU/ml, using non-inoculated microcosms as controls. A pipette was inserted into the 10-cm water column to a depth

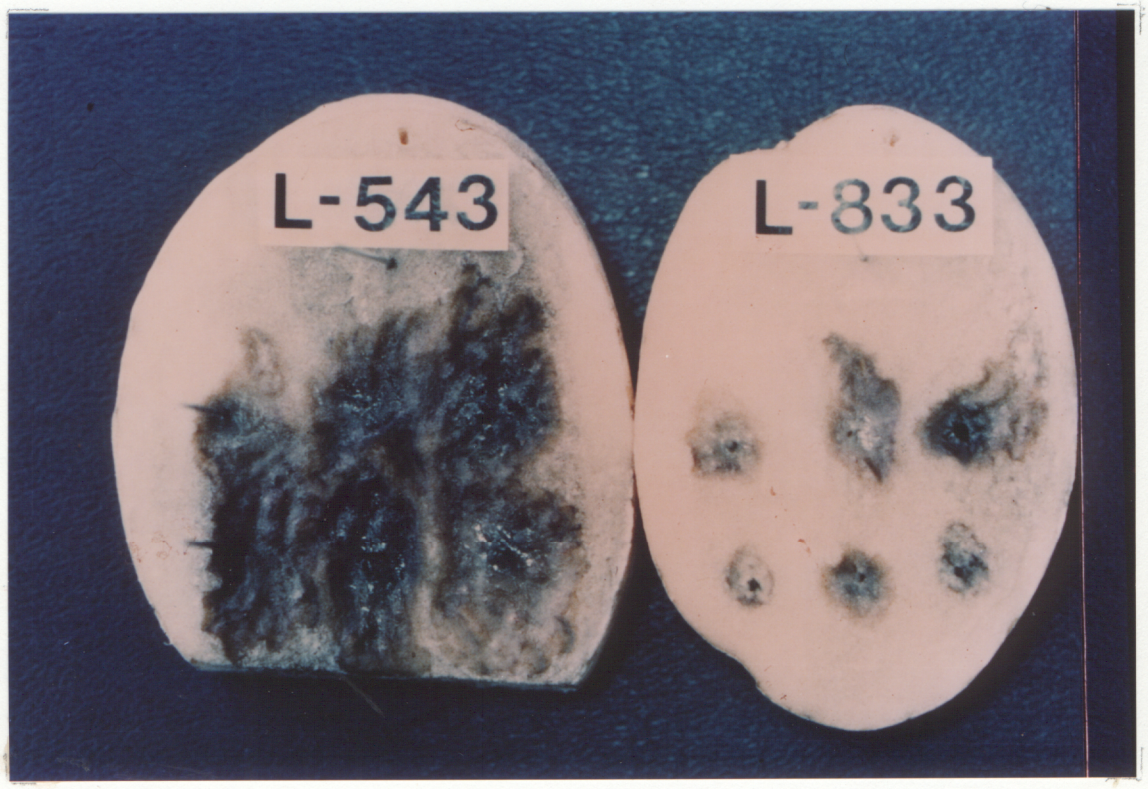

FIG. 1. Potato tuber tissue inoculated with the wildtype pathogen (L-543) and genetically-engineered strain (L-833). Tuber slices were inoculated with eye-of-the-needle method (21).

approximately 2 cm beneath the surface of the water, and a 1.0 or 0.1-ml sample was removed. Sediment samples were obtained with a 10-ml serological plastic pipet with its conical tip removed. The pipet was inserted through the depth of the sediment, and a sediment core was removed. The bottom 1.0 ml of the sediment core was used. Sample points were determined in a preliminary experiment that indicated increase in *E. carotovora* density at the water-sediment interface after inoculation. Microcosms were sampled at 0, 1, 2, 4, 8, 16, 32, and 64 days after inoculation.

Media. Samples were diluted serially and plated on appropriate media. Genetically-engineered *E. carotovora* were enumerated using plate count agar (PCA) fortified with 40 μg/ml kanamycin monosulfate and 150 μg/ml rifampicin. Wildtype strain L-863 was enumerated on PCA amended with 150 μg/ml rifampcin. Total bacteria were enumerated on 1/10 PCA (with 13.5 g/l added Bactoagar), because most colonies were recovered at this dilution of the medium. Numbers of proteolytic bacteria were estimated on Colwell YE medium (22). A starch agar was used to determine the number of amylolytic bacteria (22). Pectolytic bacteria were isolated on a modified crystal violet pectate medium (8), without the addition of crystal violet and sodium dodecyl sulfate. Densities of *E. carotovora* were estimated from plates incubated at 30°C for 48 h. Total bacterial densities were estimated from plates incubated at 20°C for 96 h. Densities of amylolytic, proteolytic, and pectolytic bacteria were estimated from plates incubated at 20°C for 48 h. Counts were expressed as CFU/ml of water or sediment.

Experimental design and statistical analysis. A randomized complete block experimental design was used; experiments were blocked over time. Replicate samples on the same day were precluded due to the large numbers of plates required. Therefore, three blocks were established, each consisting of 7 microcosms: a control microcosm, microcosms inoculated with genetically-engineered *E. carotovora* (10^3, 10^6, and 10^9 CFU/ml), and microcosms inoculated with wildtype *E. carotovora* (10^3, 10^6, and 10^9

CFU/ml). In the statistical analysis, log transformed values of bacterial densities have been used. A three-way analysis of variance (ANOVA) was used to evaluate statistical significance between inoculated strains, inoculation doses, and substrates for *E. carotovora* density on individual sampling days. A two-way ANOVA was used to evaluate statistical significance among inoculation treatments and substrates for densities of total, proteolytic, pectolytic and amylolytic bacteria.

RESULTS

Strain effects. Densities of genetically-engineered and wildtype *E. carotovora* declined in the water and sediment of aquatic microcosms (Fig. 2). On individual sampling days, the densities of the two strains in water or in sediment were not significantly different ($p > 0.05$).

Dose effects. Up to 8 days after inoculation, *E. carotovora* density differed significantly among microcosms inoculated with different doses of bacteria ($p < 0.05$; Fig. 2). However, by day 16, differences in *E. carotovora* density between microcosms initially inoculated with doses of 10^6 and 10^9 cells/ml were not statistically significant ($p > 0.05$).

Partitioning. We observed a shift in distribution and partitioning of genetically-engineered and wildtype *E. carotovora* from the water column into the sediment (Fig. 2). Four to 16 days after inoculation into the water column, density of *E. carotovora* in the sediment was significantly higher than in the water column ($p < 0.05$).

Effect on total bacteria. The density of total bacteria is presented in Figure 3. In microcosms inoculated with 10^9 CFU/ml, total bacterial density was not distinguished from *E. carotovora* density, until the latter declined below the density of total indigenous bacteria. Total bacteria were significantly higher in microcosms treated with added bacteria for 16 days ($p < 0.05$). These levels were maintained after the decline of genetically-engineered and wildtype *E. carotovora* strains. However, 32 and 64 days af-

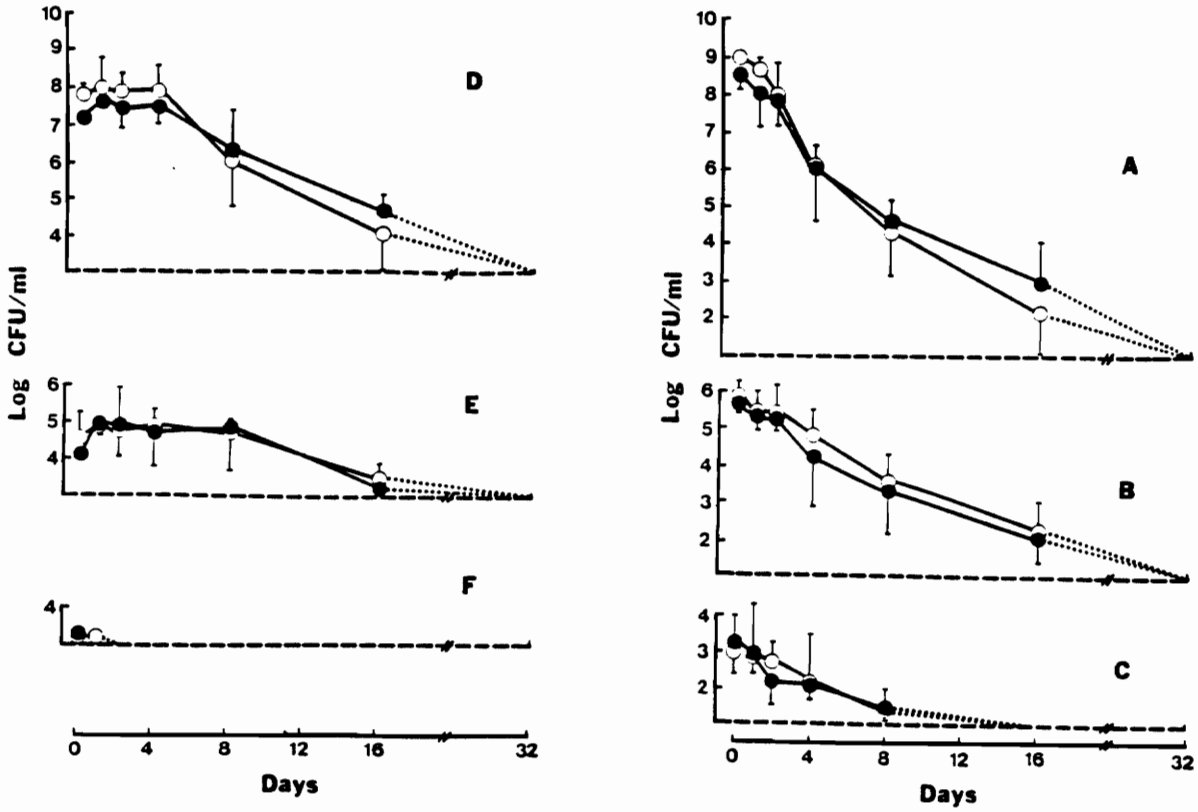

FIG. 2. Survival of *E. carotovora* in water (A, B, C) and sediment (D, F, F) of aquatic microcosms. Initial inoculation levels were 10^9 (A and D), 10^6 (B and E), and 10^3 (C and F) CFU/ml. (●) indicates genetically-engineered strain (L-864), and (○) indicates wildtype strain (L-863). Dashed lines represent minimum levels of detection. Dotted lines connect points to observation times at which no colonies were observed. Error bars represent one standard deviation unit and are included within the symbol when not shown.

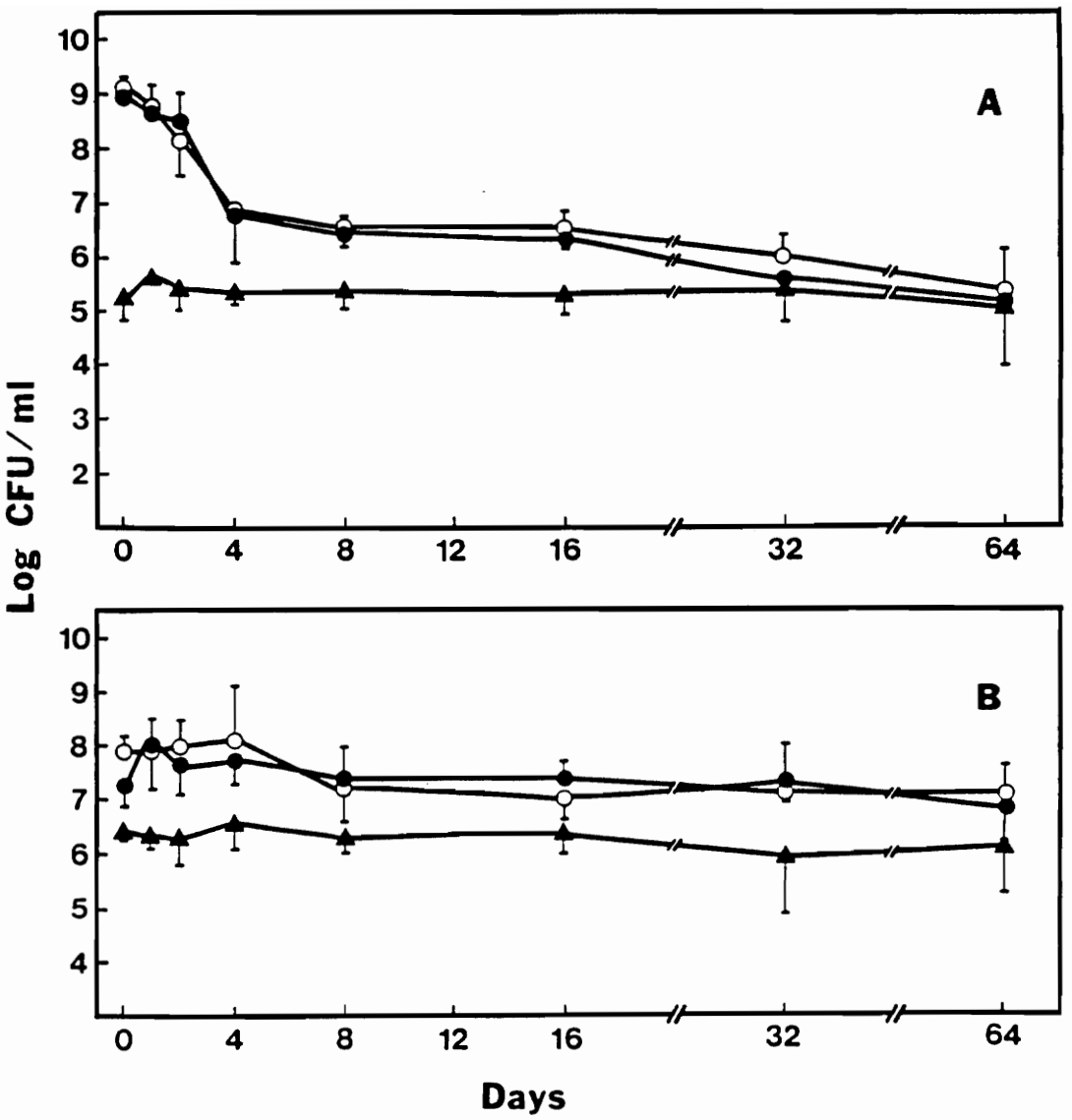

FIG. 3. Density of total bacterial populations in water (A) and sediment (B), in microcosms inoculated with *Erwinia carotovora* genetically-engineered strain (●), wildtype strain (○), and in non-inoculated microcosms (▲). Error bars represent one standard deviation unit and are included within the symbol when not shown.

ter inoculation differences were not significant ($p = 0.059$, $p = 0.873$, respectively). The total bacterial density was not statistically different in microcosms inoculated with genetically-engineered and wildtype strain ($p > 0.05$).

Effect on proteolytic bacteria. Densities of proteolytic bacteria are shown in Figure 4. Because of the ability of the genetically-engineered and wildtype strains to degrade proteins, the initial density of proteolytic bacteria of 10^9 CFU/ml includes *E. carotovora* (2). Density of bacteria capable of degrading proteins remained significantly elevated compared to control microcosms for 16 days ($p < 0.05$), even after *E. carotovora* density declined. Differences in inoculated and control microcosms were not significant at days 32 and 64 ($p > 0.05$).

Effect on pectolytic bacteria. Both *E. carotovora* strains have the ability to degrade pectate. Similar to proteolytic bacterial densities, the density of pectolytic bacteria in inoculated microcosms includes genetically-engineered or wildtype *E. carotovora.* (Fig. 5). Following the decline of *E. carotovora* strains in microcosms treated with added bacteria, the density of pectolytic bacteria was not significantly different in the treated microcosms compared to control microcosms ($p > 0.05$).

Effect on amylolytic bacteria. The density of starch utilizing bacteria (i.e., amylolytic bacteria) is shown in Figure 6. We observed no apparent difference in density of amylolytic bacteria in inoculated compared to non-inoculated microcosms ($p > 0.05$).

DISCUSSION

After introduction to aquatic microcosms, densities of genetically-engineered and wildtype *E. carotovora* declined. This finding confirms previous reports that bacterial populations decline following introduction into a system (25, 27, 29) and further supports Alexander (1), Liang et al. (16), and Mallory et al. (17) that microbial climax communities seem to resist invasions by introduced exogenous organisms. In a related

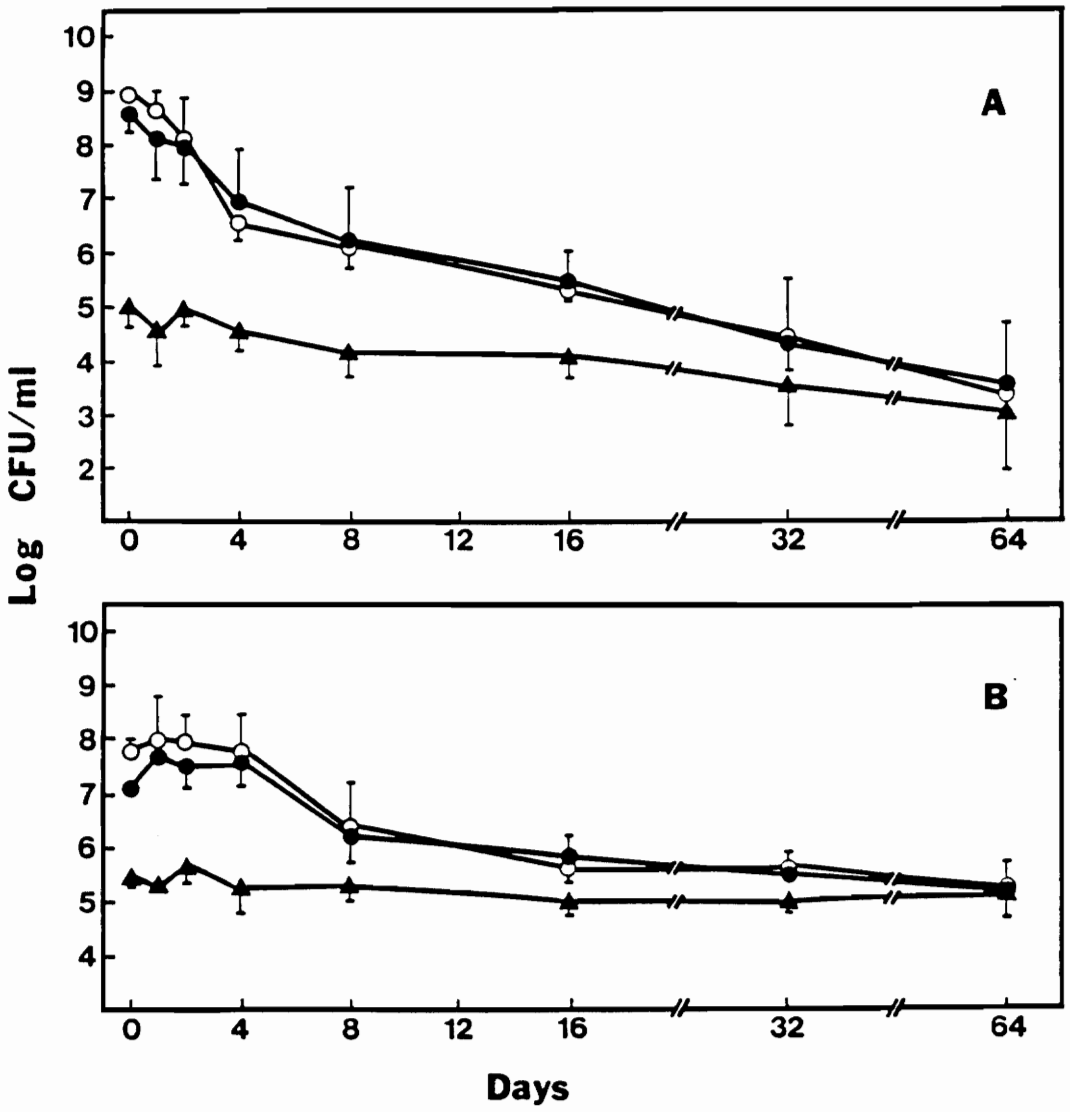

FIG. 4. Density of proteolytic bacteria in water (A) and sediment (B), in microcosms inoculated with *Erwinia carotovora* genetically-engineered strain (●), wildtype strain (○), and in non-inoculated microcosms (▲). Error bars represent one standard deviation unit and are included within the symbol when not shown.

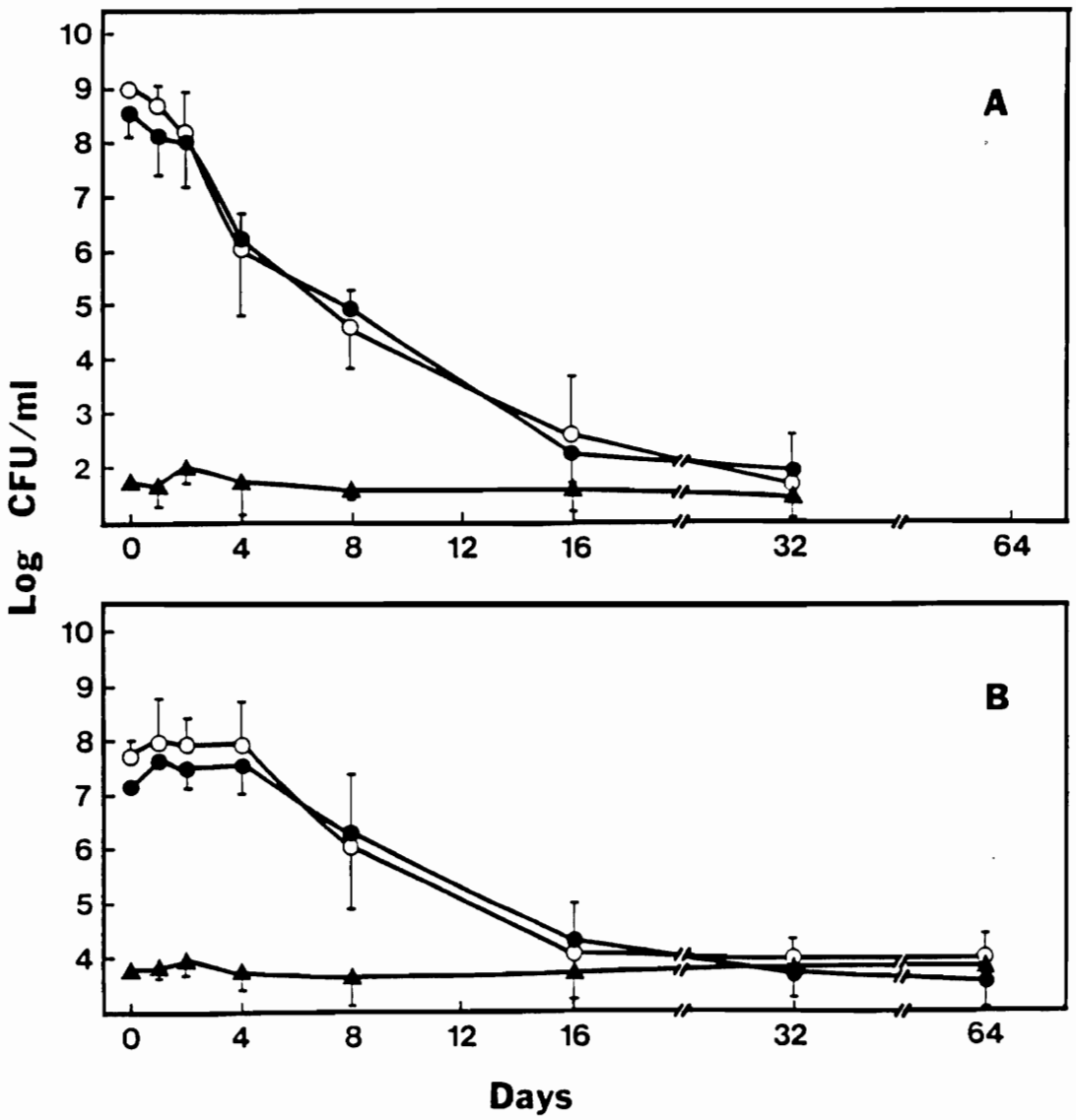

FIG. 5. Density of pectolytic bacteria in water (A) and sediment (B), in microcosms inoculated with *Erwinia carotovora* genetically-engineered strain (●), wildtype strain (○), and in non-inoculated microcosms (▲). Error bars represent one standard deviation unit and are included within the symbol when not shown.

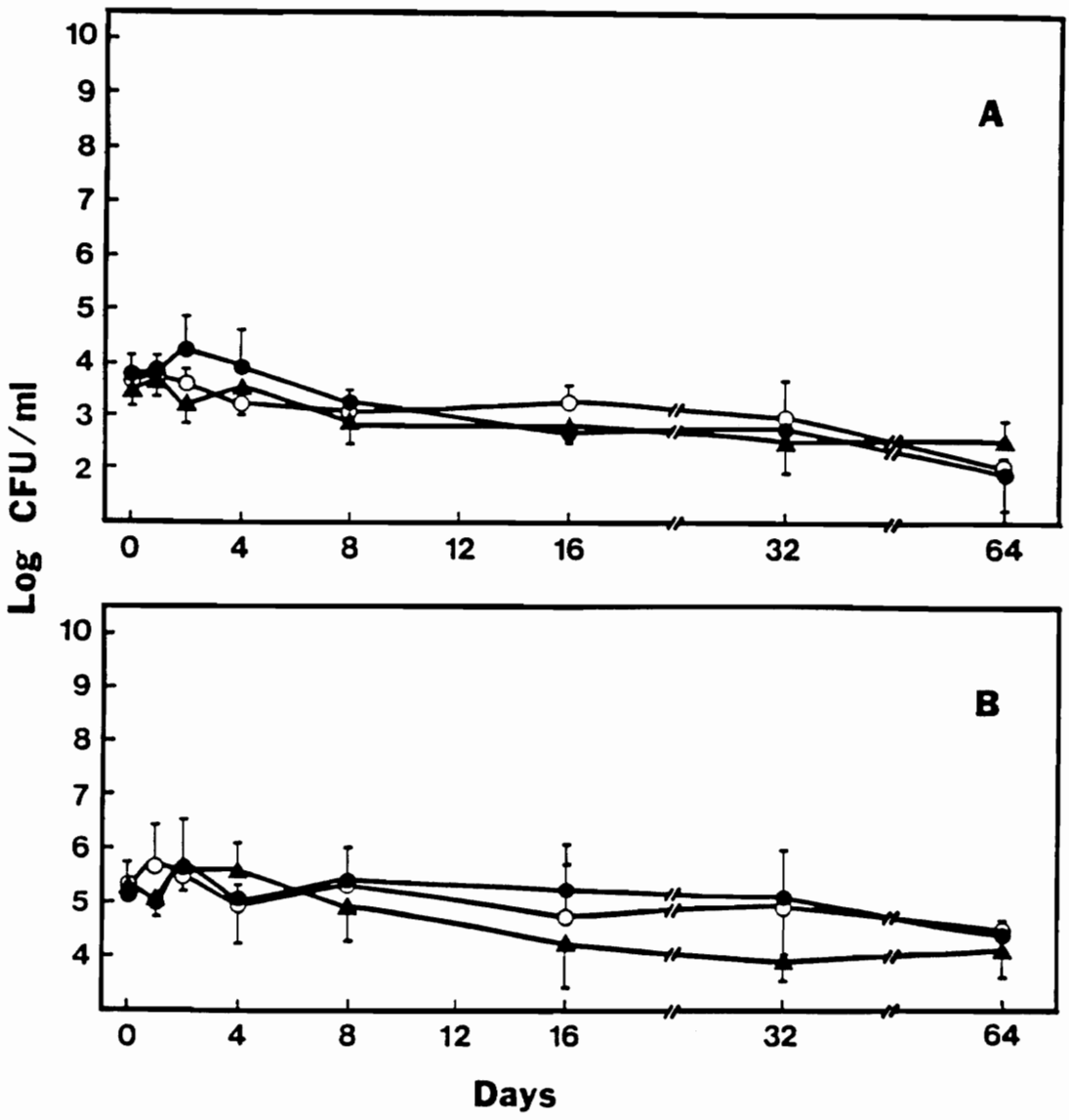

FIG. 6. Density of amylolytic bacteria in water (A) and sediment (B), in microcosms inoculated with *Erwinia carotovora* genetically-engineered strain (●), wildtype strain (○), and in non-inoculated microcosms (▲). Error bars represent one standard deviation unit and are included within the symbol when not shown.

study, genetically-engineered *E. carotovora* did not survive in soil microcosms (data not shown). It is known that bacterial populations decrease in numbers as well as activity when conditions are not optimal (23). *Erwinia carotovora* is a plant pathogen, and thus the decline of genetically-engineered and wildtype strains in aquatic microcosms that do not contain intact host plants is not surprising. Other factors may influence the decline of *E. carotovora* in the water and sediment of aquatic microcosms, such as competition with indigenous bacteria or feeding by higher trophic organisms (e.g., protozoa or nematodes) (12, 16, 17, 19, 24).

Genetically-engineered and wildtype *E. carotovora* declined at the same rate both in water and in sediment. This indicates similar physiological and ecological characteristics of both strains. Evidently, superior ability to rot living plant tissue does not confer the wildtype with a selective adaptation for survival in pond sediments or water. This trait might faciliate differences in plant tissue. Sixteen days after inoculation of 10^6 and 10^9 CFU/ml *E. carotovora* into aquatic microcosms, their density decreased to the similar levels in water (Fig. 2A,B) and sediment (Fig. 2D,F). Thus, *E. carotovora* survival was not enhanced or maintained by a heavier dose of potential growth substrates derived from cell lysis in microcosms inoculated with higher than lower bacterial densities. These findings contradict observations by Postgate (20) of such cryptic growth for the introduced bacteria.

Jorge and Harrison (13) suggested that niches exist in aquatic systems (such as stream bed sediments) where the wildtype *E. carotovora* may multiply and release cells into flowing water. We observed a shift in the spatial distribution of *E. carotovora* shortly after introduction to aquatic microcosms. The density of *E. carotovora* increased in microcosm sediment, probably due to the sedimentation, increased nutrient availability in sediment, and successful competition at low oxygen tension (*E. carotovora* is a facultative anaerobe).

The introduction of genetically-engineered *E. carotovora* affected the indigenous bacterial community. Density of total bacteria significantly increased, as well as the density of bacteria belonging to proteolytic functional group, indicating intensive protein degradation in inoculated microcosms. The observed increase can be attributed to the "inoculum-nutrient" effect; inoculated cells of *E. carotovora* died off and became a nutrient source for indigenous bacterial populations. These findings support cryptic growth for indigenous proteolytic bacteria (20). In contrast, the density of indigenous pectolytic and amylolytic bacteria was not affected by the introduction of *E. carotovora.*

We observed no discernable differences in the survival of genetically-engineered or wildtype *E. carotovora* in aquatic microcosms. Both populations declined at a similar rate, and their densities fell below the level of detection in 32 days (Fig. 2). On one hand, a population of *E. carotovora* that persists in low but undetectable densities could remain a potential threat if conditions became more favorable and the density of genetically-engineered *E. carotovora* increased. On the other hand, risk appears to be slight, because genetically-altered *E. carotovora* did not displace indigenous bacteria capable of starch, protein, or pectate degradation.

The absence of persistence of genetically-engineered *E. carotovora* in aquatic microcosms should be interpreted with caution. While an organism may not be established in the microcosm, it does not follow that it will not be established in the ecosystem that has a greater degree of physical and biological heterogenity (30). Prior to the actual release of genetically-engineered *E. carotovora,* additional tests should be made in microcosms simulating perturbed or simplified environments that are most susceptible to invasions by introduced organisms.

ACKNOWLEDGMENTS

This study was supported by the E. I. duPont de Nemours and Company Educational Foundation; Mobil Foundation; grant BSR-8705445 from the National Science Foundation; and by grant R-813805-01-0 from the Environmental Protection Agency.

We are grateful to H. Kay Austin for providing helpful comments on the draft, and to Darla Donald for preparing the manuscript for publication.

LITERATURE CITED

1. **Alexander, M.** 1971. Microbial ecology. John Wiley & Sons, Inc., New York.
2. **Allen, C., V. K. Stromberg, F. D. Smith, G. H. Lacy, and M. S. Mount.** 1986. Complementation of an *Erwinia carotovora* subsp. *carotovora* protease mutant with a protease-encoding cosmid. Mol. Gen. Genet. **202**:276-279.
3. **Bartz, J. A.** 1982. Infiltration of tomatoes immersed at different temperatures to different depths in suspensions of *Erwinia carotovora* subsp. *carotovora*. Plant Dis. **66**:302-306.
4. **Buikema, A. L., Jr., J. D. Miller, and W. H. Yongue, Jr.** 1978. Effects of algae and protozoans on the dynamics of *Polyarthra vulgaris*. Verh. Internat. Verein. Limnol. **20**:2395-2399.
5. **Cairns, J., Jr., and J. R. Pratt.** 1986. Ecological censequence assessment: effects of bioingeneered microorganisms. Water Res. Bull. **22**:171-182.
6. **Chakrabarty, A. M., J. S. Karns, J. J. Kilbane, and D. K. Chatterjee.** 1984. Selective evolution of genes for enhanced degradation of persistent, toxic chemicals, p. 43-54. *In* W. Arber, K. Illmensee, W. J. Peacock, and P. Starlinger (ed.), Genetic manipulation: Impact on man and society. Cambridge University Press, New York.

7. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Ann. Rev. Phytopathol. 34:645-676.

8. **Cuppels, D., and A. Kelman.** 1974. Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue. Phytopathology **64**:468-475.

9. **Fiksel, J. R., and V. T. Covello.** 1986. The suitability and applicability of risk assessment methods for environmental applications of biotechnology, p. 1-34. *In* J. Fiksel, and V. T. Covello (ed.), Biotechnology risk assessment: Issues and methods for environmental introductions. Pergamon Press, New York.

10. **Gorden, R. W.** 1972. Field and laboratory microbial ecology. W. C. Brown Company Publishers, Dubuque, Iowa.

11. **Harrison, M. D., G. D. Franc, D. A. Maddox, J. E. Michaud, and N. J. McCarter Zorner.** 1987. Presence of *Erwinia carotovora* in surface water in North America. J. Appl. Bacteriol. **62**:565-570.

12. **Ingham, R. E., J. A. Trotymow, E. R. Ingham, and D. C. Coleman.** 1985. Interactions of bacteria, fungi, and their nematode grazers: effects on nutrient cycling and plant growth. Ecol. Monogr.**55**:119-140.

13. **Jorge, P. E., and M. D. Harrison.** 1986. The association of *Erwinia carotovora* with surface water in Northeastern Colorado. I. The presence and population of the bacterium in relation to location, season and water temperature. Am. Potato J. **63**:517-531.

14. **Kotoujansky, A.** Molecular genetics of pathogenesis by soft-rot erwinias. Ann. Rev. Phytopathol. **25**:405-430.

15. **Lacy, G. H., R. C. Lambe, and C. M. Berg.** 1981. Iris soft rot caused by *Erwinia chrysanthemi,* assosiated with overhead irrigation and its control by chlorination. Vol. 31, Int. Plant Propagators' Soc. Combined Proceedings for 1981.

16. **Liang, L. N., J. L. Sinclair, L. M. Mallory, and M. Alexander.** 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Applied Env. Micro. 44:708-714.

17. **Mallory, L. M., J. L. Sinclair, L. N. Liang, and M. Alexander.** 1982. A simple and sensitive method for assessing survival in environmental samples of species used in recombinant DNA research. Recombinant DNA Technol. Bull. 5:5-6.

18. **McCarter-Zorner, N. J., G. D. Franc, M. D. Harrison, J. E. Michaud, C. E. Quinn, I. A. Sells, and D. C. Graham.** 1984. Soft rot *Erwinia* bacteria in surface and underground waters in southern Scotland and in Colorado, United States. J. Appl. Bacteriol. 57:95-105.

19. **Porter, K. G., E. B. Sherr, M. Pace, and R. W. Sanders.** 1985. Protozoa in planktonic food webs. J. Protozool. 32:409-415.

20. **Postgate, J. R.** 1976. Death in macrobes and microbes, p. 1-19. *In* T. R. G. Gray, and J. R. Postgate (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge.

21. **Roberts, D. P., P. M. Berman, C. Allen, V. K. Stromberg, G. H. Lacy, and M. S. Mount.** 1986. Molecular cloning of a 3.4 kilobase DNA fragment mediating production of pectate lyases. Can. J. Plant Pathol. 8:17-27.

22. **Rodina, A. G.** 1965. Methody vodnoi microbiologii. Translated 1972 as Methods in aquatic microbiology. R. Colwell and M. S. Zambruski (ed.). University Park Press, Baltimore.

23. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51:365-379.

24. **Sambanis, A., and A. G. Frederickson.** 1988. Persistence of bacteria in the presence of viable, nonencysting, bacteriovorous ciliates. Microb. Ecol. 16:197-211.

25. **Sharples, F. E.** 1983. Spread of organisms with novel genotypes: Thoughts from an ecological perspective. Recombinant DNA Technol. Bull. **6**:43-56

26. **Simberloff, D.** 1985. Predicting ecological effects of novel organisms: Evidence from higher organisms, p. 152-161. *In* H. O. Halvorson, D. Pramer, and M. Rogul (ed.), Engineered organisms in the environment: Scientific issues. American Society for Microbiology, Washington, D.C.

27. **Stacey, G.** 1985. The *Rhizobium* experience, p. 109-121. *In* H. O. Halvorson, D. Pramer, and M. Rogul (ed.), Engineered organisms in the environment: Scientific issues. American Society for Microbiology, Washington, D.C.

28. **Stotzky, G., and H. Babich.** 1984. Fate of genetically-engineered microbes in natural environments. Recombinant DNA Technol. Bull. 7:163-188.

29. **Strauss, H. S., D. Hattis, G. Page, K. Harrison, S. Vogel, and C. Caldarf.** 1984. Genetically-engineered microorganisms: II. Survival, multiplication and genetic transfer. Recombinant DNA Technical Bull. **9**:69-88.

30. **Suter, G. W., II.** 1985. Application of environmental risk analysis to engineered organisms, p. 211-219. *In* H. O. Halvorson, D. Pramer, and M. Rogul (ed.), Engineered organisms in the environment: Scientific issues. American Society for Microbiology, Washington, D.C.

31. **Wetzel, R. G.** 1983. Limnology, 2nd ed. W.B. Saunders, Philadelphia.

Chapter 2

Enumerating Low Densities of Genetically-Engineered *Erwinia carotovora* in Soil *

ABSTRACT

Ability to detect genetically-engineered microorganisms (GEMs) present in low densities in environmental samples is important to evaluate any risk in their release. We developed an inexpensive, quantitative, and sensitive technique for detection of genetically-engineered *Erwinia carotovora* in soil samples. Enrichment media, antibiotic resistance, and most probable number (MPN) analysis were used to enumerate as few as 1 to 10 target cells/10 g soil. The MPN technique strongly correlated with selective plating in estimation of

* This chapter was published in short form in Let. Appl. Microbiol. 10:55-59, with the authors V. S. Scanferlato, D. R. Orvos, G. H. Lacy, and J. Cairns, Jr.

GEMs density in soil samples ($p < 0.001$, $r^2 = 0.975$). Recovery of GEMs from soil was significantly higher using the MPN compared to the selective plating technique ($p < 0.001$). Percentage of GEMs recovered by MPN from soil inoculated with 10^0 CFU/g to 10^7 CFU/g was independent of inoculum density. The MPN technique was used to assess the fate of GEMs introduced into soil microcosms; genetically-engineered *E. carotovora* declined at a rate of 1.2 log units/g soil/10 days. Because of its high sensitivity and selectivity, the MPN technique can be used to monitor populations of genetically-engineered *E. carotovora* at low densities in soil after their environmental release.

INTRODUCTION

The application of genetically-engineered microorganisms (GEMs) is imminent due to apparent economic benefits. Detection of GEMs is, on one hand, critical in ascertaining whether introduced GEMs persist long enough to carry out commercially desired tasks (22). On the other hand, since the potential negative ecological impact of GEMs depends upon their establishment in the environment (2, 5, 30), the ability to detect GEMs in low population densities is "conditio sine qua non" for evaluating the risks of their environmental release (8, 22).

Currently used detection methods make it difficult to distinguish between cases of "die out" (death) and "die back" (decline of populations of GEMs below detectable levels). The DNA hybridization and DNA amplification techniques developed to enumerate GEMs permit detection of as few as 1 to 100 cells/g soil or sediment (13, 28). However, these methods are expensive both in equipment and expendable materials. Less sensitive plating techniques often do not detect cells in densities lower than about 10^3 cells/g soil.

We describe a sensitive and inexpensive technique, coupling enrichment and most probable number (MPN) analysis, to enumerate genetically-engineered *Erwinia*

carotovora in soil samples. The GEM constructed from *Erwinia carotovora* subsp. *carotovora*, a causative agent of soft-rot in plants, was chosen for this study because of its potential ecological and economic significance as a biological control agent. Phenotypic characteristics of genetically-engineered *E. carotovora* that enable its culture in enrichment medium are (a) ability of the GEM to produce pectolytic enzymes and to use sodium polypectate as a carbon source, and (b) resistance to rifampicin and kanamycin monosulfate, which permits the growth of the GEM in the presence of these antibiotics.

Density of genetically-engineered *E. carotovora* in the soil was determined by introducing samples into a series of tubes containing the enrichment medium and recording the number of tubes showing growth of the target organism. Most probable number analysis was applied to make a mathematical inference about GEMs viable count based on the number of positive and negative tubes. It was assumed that all viable GEMs grew in the enrichment medium and that they were randomly distributed in diluted samples (6, 20). The MPN method and its applications have been described previously (1, 6, 14, 20, 21, 26, 27).

The objective of this research was to develop a sensitive and inexpensive technique for enumerating genetically-engineered *E. carotovora* in low density in soil samples. This technique can be used to monitor the persistence of genetically-engineered *E. carotovora* after their release, both in terrestrial and aquatic environments.

MATERIALS AND METHODS

Bacterial strains. The genetically-engineered strain L-863 was constructed from wildtype *E. carotovora* strain L-543. An extracellular pectate lyase on a plasmid was disarmed *in vitro* by deletion mutation, and a DNA fragment from Tn*903* conferring kanamycin resistance was inserted. Part of the engineered plasmid was chromosomally

inserted in the pathogen's chromosome in a *cis* relationship to the wildtype pectate lyase gene (3). The spontaneous rifampicin mutant of L-833, designated L-834, was used in the study. L-834 is resistant to 150 mg/l rifampicin and 40 mg/l kanamycin, and 30% reduced in pathogenicity compared to the wildtype. For growth and biochemical characteristics tested, the wildtype L-543 and genetically-engineered l-864 are identical (data not shown).

Genetically-engineered *E. carotovora* was grown for 24 h in nutrient broth at 30°C to a density of approximatelly 10^9 CFU/ml, estimated turbidiometrically at 550 nm. Cells were harvested by centrifugation at 10,000x*g* for 15 min. The pellet was resuspended in sterile distilled water, and the procedure repeated. Appropriate dilutions were made in sterile distilled water.

Inoculation of soil microcosms. Simple microcosms were constructed from 850 ml glass Mason jars containing 300 g of soil that was collected at the Virginia Polytechnic Institute and State University farm at Whitethorn. Soil was a Hayter loam type and consisted of 39% sand, 41% silt, and 20% clay. The soil was passed through a 2-mm sieve and air dried. Thirty 30 ml of inoculum were added to 300 g of soil to provide approximatelly (a) 10^6 CFU/g soil to assess the recovery of bacteria adhered to soil particles, (b) 10^3 to 10^8 CFU/g soil to determine the relationship between numbers of GEMs obtained by selective plating and MPN technique, (c) 10^0 to 10^7 CFU/g soil (ten-fold dilutions) to determine the recovery efficiencies of selective plating and MPN technique, or (d) 10^4 CFU/g soil to study GEM survival at low densities. Numbers of inoculated cells were determined by plating on selective medium amended by appropriate antibiotics and expressed as log CFU/g dry soil.

Selective plating. Selective medium used for plating of genetically-engineered *E. carotovora* was plate count agar (PCA) amended with 150 mg/l rifampicin and 40 mg/l kanamycin monosulfate. A 0.1-ml aliquot of diluted soil was plated in triplicate and

incubated at 30°C for 48 h. Plates with 20 to 400 colonies were counted. To reduce the detection limit, soil samples containing 10^3 and 10^4 CFU of genetically-engineered *E. carotovora* /g soil were diluted, and 0.2-ml aliquots were plated on each of six plates.

Recovery of adhered bacteria. We assessed the efficiency of sonication and shaking to remove bacteria adhered to soil particles. Four 1.0-g samples were used for each treatment. Soil was added to 10- or 90-ml sterile water for sonication or shaking, respectively. Samples were sonicated for 5, 15, or 45 s at 5 W using Ultrasonic Homogenizer (4710 Series, Cole-Parmer Instrument Co., Chicago, Illinois 60648) or shaken on an orbital shaker at 200 rpm for 5, 15, or 45 min. Aliquots of each treatment were plated on selective medium amended with antibiotics. The highest recovery was achieved with 45 min shaking (22.7% of inoculated bacteria). According to Fisher's LSD test, the number of CFU recovered after 15 min shaking was not significantly different. In the next experiments, 45-min shaking preceded the other steps.

MPN technique. An enrichment medium containing sodium polypectate (23) and the appropriate antibiotics (40 mg/l kanamycin monosulfate, 150 mg/l rifampicin, 75 mg/l cycloheximide) was used to culture genetically-engineered *E. carotovora* from soil samples. The amount of soil used, its dilution, and the choice of container ("tube") in which the soil-enrichment medium mixture was incubated (microtiter plates, 10- and 20-ml tubes, or milk dilution bottles) depended upon the expected density of GEMs in soil.

An appropriately diluted soil suspension was introduced into 10 tubes, and an equal volume of double-concentrated enrichment medium was added. The second and third series of 10 tubes received 10- and 100-fold dilutions of original soil-water suspension, respectively. Tubes were incubated aerobically at 30°C for 96 h. The number of tubes showing the growth at each soil dilution level was recorded. Based on the number of positive and negative tubes at each dilution level, the most probable number of GEMs

per tube was determined using the table compiled by Halvorson and Ziegler (14), published in Koch (20). The density of GEMs was expressed as MPN/g soil.

Cloudiness of the medium and the change of color from orange-red to yellow always indicated the growth of GEMs. This was checked by transferring an aliquot of the soil-enrichment medium suspension onto crystal violet pectate medium (CVP) (9) using a sterile toothpick. Plates were incubated at 30°C for 48 h. Characteristic deep pits (Fig. 1) were proof of the presence of *E. carotovora* in a specific tube. The possibility that erwiniae indigenous to the sample caused pitting was eliminated by transferring colonies onto PCA fortified with antibiotics. Colonies that grew on this medium were considered to be the genetically-engineered *E. carotovora* strain L-864. Growth of the GEM could not be visually detected at high concentrations of soil in enrichment medium (1 g or more in 50 ml of medium) because soil particles made the medium appear cloudy. In such cases, presence of GEMs in specific tubes was detected by their growth on CVP medium and PCA with antibiotics. Non-inoculated soil (0.5 g) was introduced into each of 100 tubes containing enrichment medium to examine the potential appearance of "false positives." The MPN did not detect the presence of genetically-engineered *E. carotovora* in tubes containing non-inoculated soil.

Recovery efficiency by selective plating and enrichment-MPN techniques. Bacterial culture of a known, turbidiometrically-estimated density was used to make eight 10-fold inoculum dilutions. The precise number of cells in inoculum was determined by plating appropriate dilutions on PCA amended with antibiotics. Consequently, soil samples were inoculated with known densities of genetically-engineered *E. carotovora*, ranging from 10^0 to 10^7 CFU/g soil. Soil samples of 1 g were diluted, homogenized, further diluted when needed, and plated on PCA amended with antibiotics, or introduced into tubes containing enrichment medium. Recovery efficiency of plating and MPN was determined at each inoculum density. Percentage recovery of bacteria was calculated by

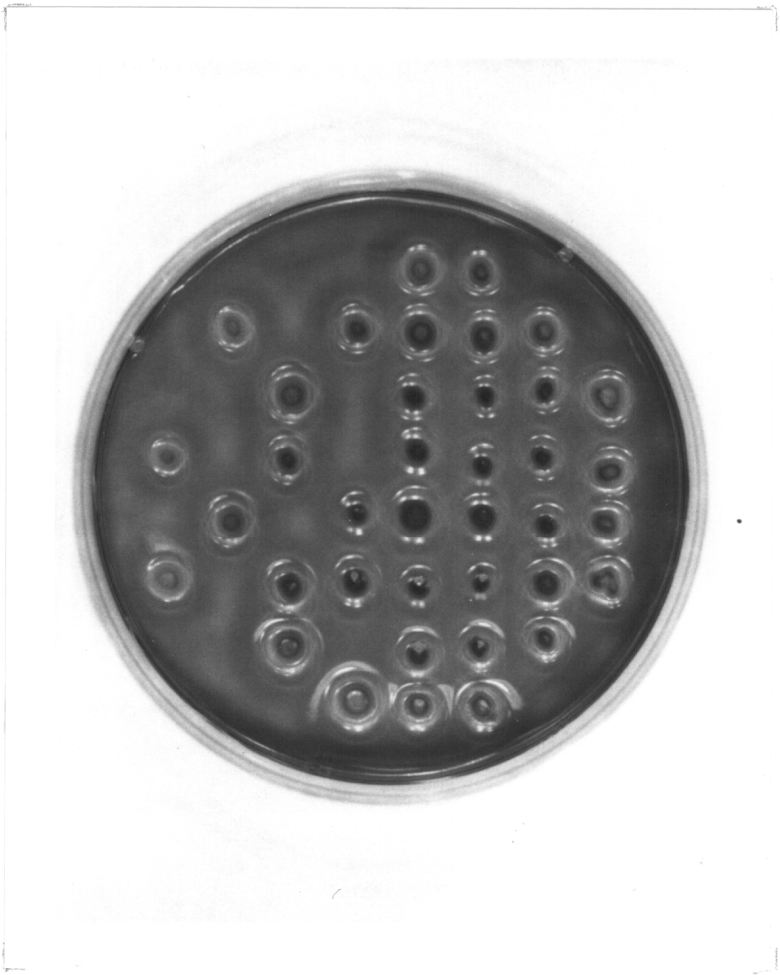

FIG. 1. Deep pits in crystal pectate violet medium (CPV) (9) indicate the growth of *Erwinia* species. Genetically-engineered *E. carotovora* strain L-864 was confirmed by transfer on antibiotic fortified medium as discussed in the text.

dividing the number of CFU recovered from soil by the number of inoculated CFU. The experiment was repeated four times.

Survival at low densities. Samples were taken from triplicate microcosms at 0, 5, 10, 15, 25, 35, 45, and 55 d after inoculation. The density of the GEMs in soil was determined by the MPN technique. Total number of non-target microorganisms was determined by plating on 1 10 PCA, since more colonies were recovered at this dilution of the medium than on undiluted PCA.

Statistical analysis. Single factor analysis of variance (ANOVA) and a multiple comparison procedure (Fisher's Least Significant Difference Test at $\alpha = 0.05$) were used to determine statistical significance among the shaking and sonication treatments. Single linear regression was used to determine correlation between: (a) log CFU estimated by selective plating and log MPN estimated by MPN technique, (b) log CFU inoculated and recovery percentage obtained by plating, and (c) log CFU inoculated and recovery percentage obtained by MPN technique. Since the correlation was not significant in (b) and (c), a paired-sample t test was used to determine statistical significance between the recovery percentages obtained by plating and by enrichment-MPN technique.

RESULTS AND DISCUSSION

We estimated simultaneously the density of genetically-engineered *E. carotovora* in soil samples by both selective plating and MPN techniques and determined the relationship between these counts. Results are shown on Figure 2. Linear correlation between the counts obtained by the two techniques was highly significant ($p < 0.001$). The MPN technique consistently estimated higher numbers of the GEMs than plating, regardless of the inoculum density (10^3 to 10^8 CFU/g soil). Figure 3 shows recovery percentages obtained by selective plating and MPN. Statistical analysis determined a significant difference between recovery efficiencies of the two techniques ($p < 0.001$).

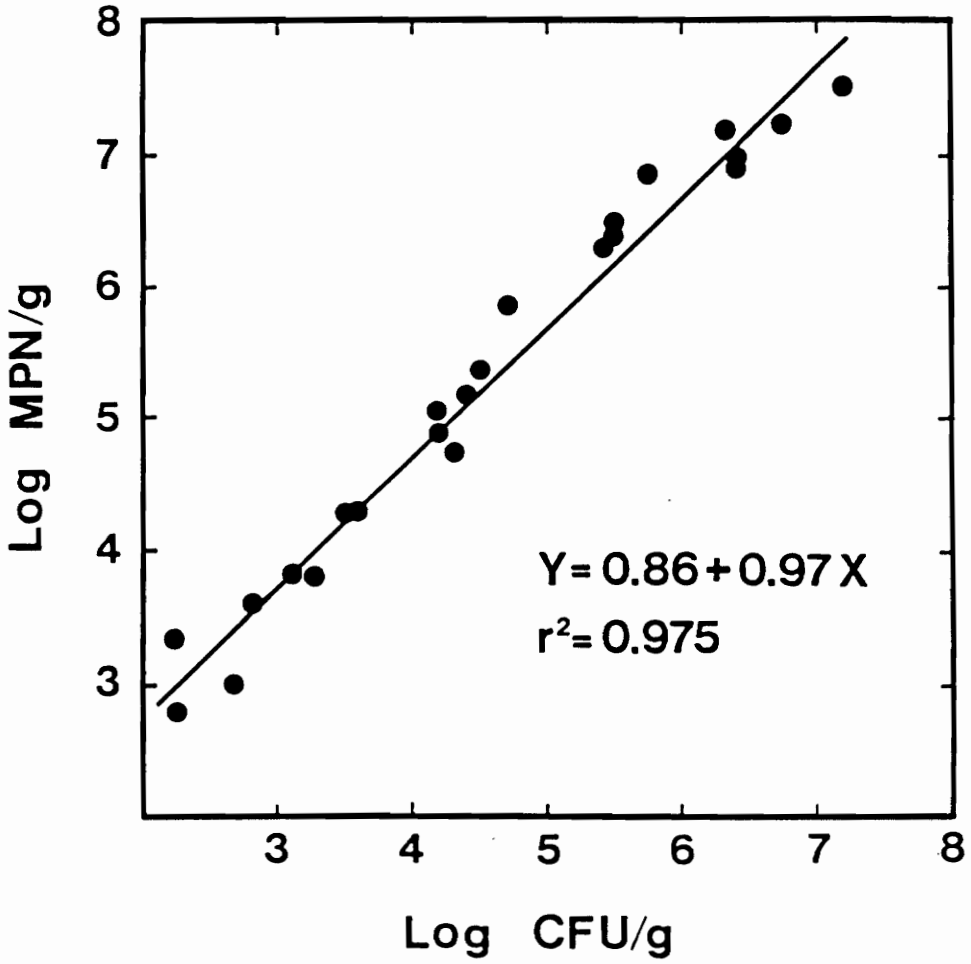

FIG. 2. Relationship between GEMs density estimated by selective plating and by MPN techniques (n = 23, F = 794.12).

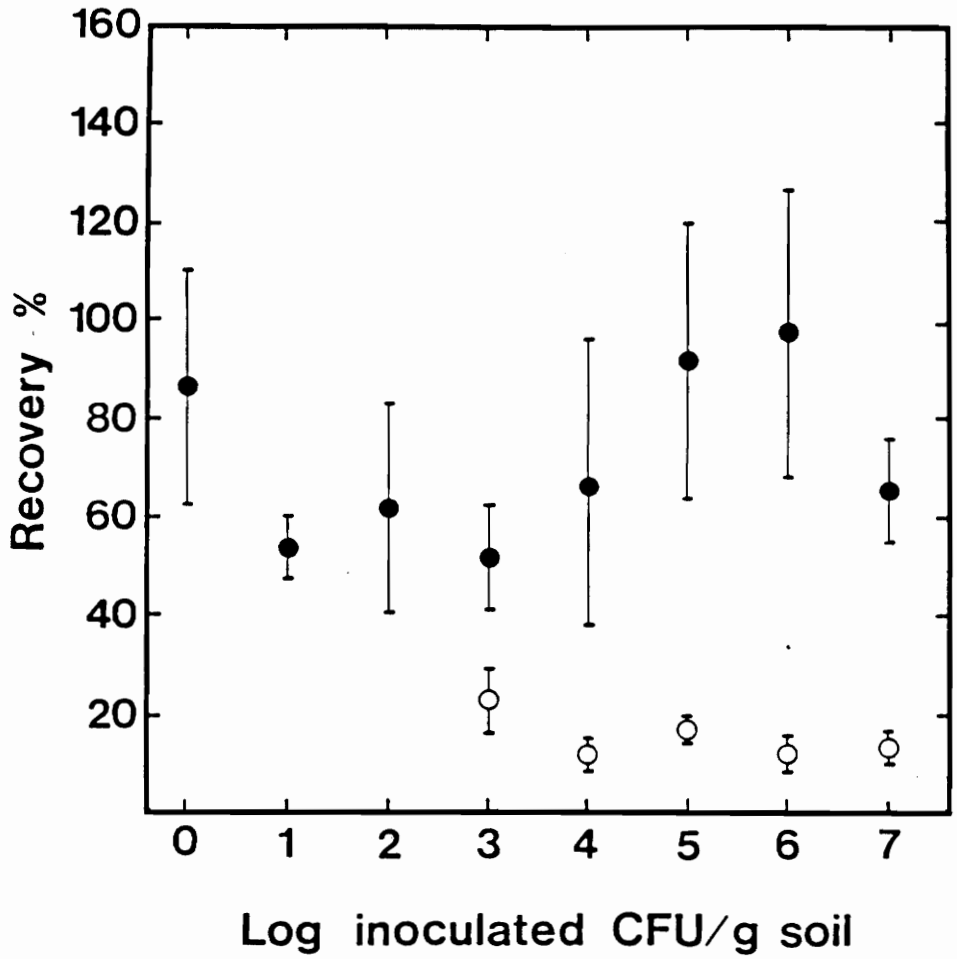

FIG. 3. Recovery efficiency of genetically-engineered *E. carotovora* from soil samples inoculated with densities ranging from 10^0 to 10^7 CFU/g dry soil. Methods of recovery were selective plating (○) and MPN (●). No GEMs were recovered by plating the soil inoculated with 10^0, 10^1, and 10^2 CFU/g. Error bars represent one standard error.

On the average, the MPN technique gave a six-fold higher estimate. Other studies that compared different methods to estimate bacterial densities also report higher values obtained by MPN than the plate count (4, 18). Jannash and Jones (18) ascribe increased counts to improved dispersion of bacterial aggregates caused by reduced medium surface tension. Presence of cells that have lost the ability to form colonies on solid media, but can still grow in suspension after addition of nutrients (7, 12, 17, 25), is another possible explanation for higher recovery of GEMs by the MPN technique.

Inadequate enrichment medium can affect bacterial counts obtained by MPN (1, 6). Alexander (1) recommended that preliminary counts be made of populations inoculated to soil in various (known) densities to determine the suitability of the selective medium. Average counts obtained by MPN in our experiment ranged between 52% and 92% of the inoculated GEMs (Fig. 3). We believe that somewhat lower numbers of GEMs recovered from soil can be attributed to bacterial losses after inoculation (24), aggregation and adherence to soil particles (22, 29), and non-culturable cells (25) rather than to an inadequate enrichment medium. Large deviation in the percentage recovery of genetically-engineered *E. carotovora* can be explained by patchy distribution of the GEMs in soil and by a low order of precision of MPN technique unless a large number of tubes is used (15, 16).

Soil samples inoculated with known densities of GEMs enabled us to check the validity of estimates obtained by the MPN technique. This was crucial at densities below 10^3 cells/g soil when direct comparison of MPN with selective plating was not possible. In soil samples inoculated with a known density of 146.3 cells/g, MPN estimated 91.9 ± 62.7 cells/g (mean$\pm$SD). The most probable number of GEMs in samples inoculated with 14.6 and 1.46 cells/g were 7.9 ± 1.9 and 1.2 ± 0.7 cells/g, respectively. This experiment demonstrated that the MPN technique can be used successfully for enumeration of the GEM in densities as low as 1 cell/g soil, regardless of inoculum

density, since the association between inoculum density and recovery efficiency was not significant ($n = 18$; $F = 0.98$; $p > 0.5$). Postma (24) reported that the inoculation density of soil did not affect the recovery efficiency; this report is in accordance with our results.

High recovery rates of the genetically-engineered *E. carotovora* obtained in our experiments do not necessarily imply that a similar recovery is possible under field conditions. It has been suggested that starvation causes better bacterial adherence to surfaces (10, 19). Moreover, stressed cells might be more susceptible to inhibitory components in the enrichment medium (e.g., antibiotics) (11).

We applied MPN to assess the fate of genetically-engineered *E. carotovora* at low population densities in soil microcosms. Estimates of GEMs density during the study were carried out against 8.6×10^6 to 1.34×10^7 CFU/g soil of indigenous non-target organisms. Populations of genetically-engineered *E. carotovora* declined at an average rate of 1.2 log units/g soil/10 d (Fig. 4). The density of GEMs estimated by the MPN technique 45 d after inoculation of 10^4 CFU/g soil was 1.1 cell/10 g soil in one of the microcosms, while the GEMs could not be detected in 10-g soil samples obtained from the other two microcosms. The MPN indicated the disappearance of the GEMs in 10-g soil samples from all microcosms 55 d after inoculation. Failure to detect the GEM after culturing the soil in the enrichment medium may indicate that all viable cells "died out" in the amount of soil that was tested.

The ability of MPN to detect genetically-engineered *E. carotovora* in extremely low numbers, if the sufficient amount of soil and enrichment medium are provided, makes this technique more sensitive than direct methods of detection. Unlike direct microscopy and immunofluorescence, which may enumerate senescent cells (8, 24), MPN takes into account only those cells that are viable and culturable. However, one possible limitation to this technique is the phenomenon of viable, but non-culturable cells, such as starved or stressed bacteria that are often unable to undergo division but remain metabolically

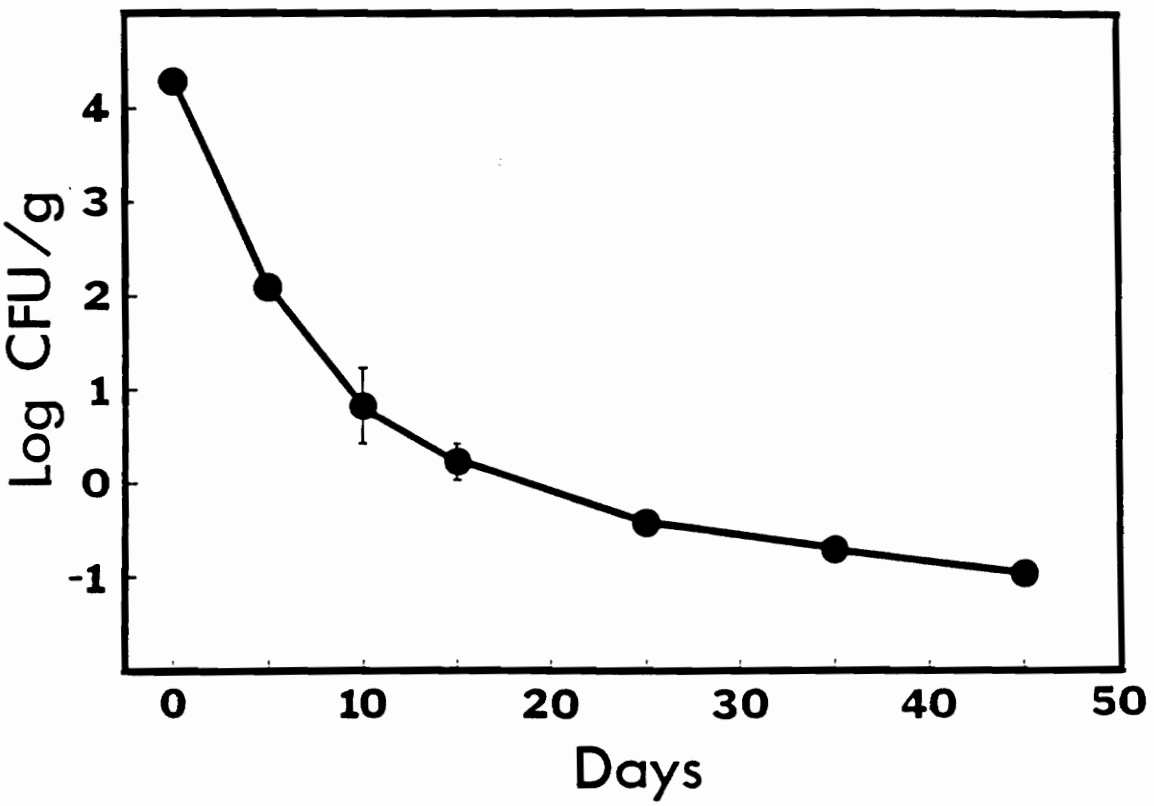

FIG. 4. Survival of genetically-engineered *E. carotovora* in soil microcosms. Error bars represent one standard error and are included within the symbol if not shown.

active (25). Non-culturable cells could cause an underestimation of bacterial density or a failure to detect target bacteria.

Culture in a selective medium detects a specific phenotype, rather than a specific genotype, which can be tracked by gene probes. However, culturing of genetically-engineered *E. carotovora* in the described enrichment medium might serve as a preliminary step for genetic methods, if techniques such as DNA amplification are not used (28).

The MPN method has some disadvantages: 1) tracking of the GEMs phenotype, but not necessarily genetically-engineered genes, 2) inefficiency from the point of view of statistics (large number of replicates are required), and 3) requirement for the growth of GEMs. Advantages of the MPN technique described here are that: 1) it is inexpensive, 2) it is selective, 3) it is sensitive, 4) it is quantitative, 5) it does not enumerate dead cells, and 6) it permits GEMs detection in environmental samples. With modification, such as a different enrichment medium, the MPN technique we describe could serve as a method to detect other GEMs as well.

ACKNOWLEDGMENTS

This study was supported in parts by the E. I. duPont de Nemours and Company Educational Foundation; Mobil Foundation; Graduate Student Assembly Development Project Fund; Grant BSR-8705445 from the National Science Foundation; and by grant R-813805-01-0 from the Environmental Protection Agency.

We wish to thank K. Austin, R. Benoit, and B. Wallace for helpful suggestions and comments, J. Yim for technical assistance, A. Giovannitti-Jensen for help in statistical analysis, and D. Donald for preparing the manuscript for publication.

LITERATURE CITED

1. **Alexander, M. 1982.** Most probable number method for microbial populations, p. 815-820. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2: Chemical and microbiological properties, 2nd ed. American Society of Agronomy, Madison, Wis.
2. **Alexander, M.** 1985. Ecological consequences: reducing the uncertainties. Issues Sci. Tech. Spring:57-68.
3. **Allen, C., V. K. Stromberg, F. D. Smith, G. H. Lacy, and M. S. Mount.** 1986. Complementation of an *Erwinia carotovora* subsp. *carotovora* protease mutant with a protease-encoding cosmid. Mol. Gen. Genet. 202:276-279.
4. **Butterfield, C. T.** 1933. Comparison of the enumeration of bacteria by means of solid and liquid media. Public Health Reports 48:1292-1297.
5. **Cairns, J., Jr., and J. R. Pratt.** 1986. Ecological consequence assessment: Effects of bioengineered microorganisms. Wat. Res. Bull. 22:171-182.
6. **Colwell, R. R.** 1979. Enumeration of specific populations by the most-probable-number (MPN) method, p. 56-61. *In* J. W. Costerton, and R. R. Colwell (ed.), Native aquatic bacteria: Enumeration, activity, and ecology, STP 695, American Society for Testing and Materials, Philadelphia.
7. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for release of genetically-engineered microorganisms. Bio/Technology 3:817-820.
8. **Colwell, R. R., C. Somerville, I. Knight, and W. Straube.** 1988. Detection and monitoring of genetically-engineered micro-organisms, p. 47-60. *In* M. Sussman, C. H. Collins, F. A. Skinner, and D. E. Stewart-Tull (ed.), The release of genetically-engineered micro-organisms. Academic Press, London.

9. **Cuppels, D., and A. Kelman.** 1974. Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue. Phytopathology 64:468-475.

10. **Dawson, M. P., B. Humphrey, and K. C. Marshall.** 1981. Adhesion: a tactic in the survival strategy of a marine vibrio during starvation. Curr. Microbiol. 6:195-198.

11. **de Boer, H. S. 1982.** Survival of phytipathogenic bacteria in soil, p. 88-108. *In* M. S. Mount, and G. H. Lacy (ed.), Phythogenic prokaryotes, vol.1. Academic Press Inc., New York.

12. **Devanas, M. A., D. Rafaeli-Eshkol, and G. Stotzky.** 1986. Survival of plasmid-containing strains of *Escherichia coli* in soil: effect of plasmid size and nutrients on survival of hosts and maintenance of plasmids. Curr. Microbiol. 13:269-277.

13. **Frederickson, J. K., D. F. Bezdicek, F. J. Brockman, and S. W. Li.** 1988. Enumeration of Tn5 mutant bacteria in soil by using a most-probable-number-DNA hybridization procedure and antibiotic resistance. Appl. Environ. Microbiol. 54:446-453.

14. **Halvorson, H. O., and N. R. Ziegler.** 1932. Application of statistics to problems of bacteriology. I. A means of determining bacterial population by the dilution method. J. Bacteriol. 25:101-121.

15. **Halvorson, H. O., and N. R. Ziegler.** 1933. Application of statistics to problems of bacteriology. II. A consideration of the accuracy of dilution data obtained by using a single solution. J. Bacteriol. 26:331-339.

16. **Halvorson, H. O., and N. R. Ziegler.** 1933. Application of statistics to problems of bacteriology. III. A consideration of the accuracy of dilution data obtained by using several dilutions. J. Bacteriol. 26:559-567.

17. **Heinmets, F., W. W. Taylor, and J. J. Lehman.** 1953. The use of metabolites in the restoration of the viability of heat and chemically inactivated *Escherichia coli.* J. Bacteriol. 67:5-14.

18. **Jannash, H. and G. E. Jones.** 1959. Bacterial populations in sea water as determined by different methods of enumeration. Limnol. Oceanogr. 4:128-139.

19. **Kjelleberg, S., B. A. Humphrey, and K. C. Marshall.** 1983. Initial phases of starvation and activity of bacteria at surfaces. Appl. Environ. Microbiol. 46:978-984.

20. **Koch, A. L.** 1981. Most probable numbers, p. 188-192. *In* P. Gerhardt (ed.), Manual of methods for general microbiology. American Society for Microbiology, Washington, D. C.

21. **Luria, S. E., and M. Delbruck.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28:491-511.

22. **McCormick, D.** 1988. Detection technology: The key to environmental microbiology. Bio/Technology 4:419-422.

23. **Meneley, J. C., and M. E. Stanghellini.** 1976. Isolation of soft-rot *Erwinia* spp. from agricultural soils using an enrichment technique. Phytopathology 66:367-370.

24. **Postma, J., J. D. van Elsas, J. M. Govaert, and J. A. van Veen.** 1988. The dynamics of *Rhizobium leguminosum* biovar *trifolii* introduced into soil as determined by immunofluorescence and selective plating techniques. FEMS Microbiol. Ecol. 53:251-260.

25. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51:365-379.

26. **Rowe, R., R. Todd, and J. Waide.** 1977. Microtechnique for moBt-probable-umber analysis. Appl. Environ. Microbiol. 33:675-680.

27. **Russek, E., and R. R. Colwell.** 1983. Computation of most probable numbers. Appl. Environ. Microbiol. 45:1646-1650.

28. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically-engineered bacteria in environmental samples. Appl. Environ. Microbiol. 54:2185-2191.

29. **Stotzky, G., and R. G. Burns.** 1982. The soil environment: clay - humus - microbe interactions, p. 105-133. *In* R. G. Burns and J. H. Slater (ed.), Experimental microbial ecology. Blackwell Scientific Publication, Boston.

30. **Strauss, H. S., D. Hattis, G. Page, K. Harrison, S. Vogel, and C. Caldarf.** 1984. Genetically-engineered microorganisms: survival, multiplication, and genetic transfer. Recombinant DNA Technol. Bull. 9:69-88.

Chapter 3

Survival of Genetically-Engineered *Erwinia carotovora* in Perturbed and Unperturbed Aquatic Microcosms and Effect on Recovery of Indigenous Bacteria *

Abstract

Genetically-engineered *Erwinia carotovora* persisted significantly longer in thermally-perturbed microcosms (35 days) than in non-stressed microcosms (5 days). Decreased pressure of competitors and predators and increased nutrient availability were examined as the most probable reasons for greater vulnerability of perturbed microcosms to colonization by genetically-engineered microorganisms (GEMs). Indigenous bacteria that competed with GEMs for the same nutrient sources (protein, cellulose, pectate) were

* This chapter has been submitted to Microbial Ecology, with the authors V. S. Scanferlato, G. H. Lacy, and J. Cairns, Jr.

present immediatelly after perturbation in densities 1 to 2 orders of magnitude lower than in unperturbed microcosms, but their populations increased to densities significantly higher than in unperturbed microcosms 10 to 15 days after inoculation. Bacterial predators (protozoans, cladocerans, nematodes, and rotifers) were present during the experiment in unperturbed microcosms, while dense populations of bacteriovorous nanoflagellates developed in perturbed microcosms. Inoculation with GEMs did not affect the recovery of total and cellulolytic indigenous populations in perturbed microcosms. However, recolonization by indigenous proteolytic bacteria seemed to be retarded in GEMs presence, possibly due to the competitive exclusion.

Introduction

Knowledge of the survival ability of genetically-engineered microorganisms (GEMs) is crucial in predicting the risk accompanying their release into the environment [11, 24]. Little is known about the factors that must be present for GEMs establishment [23]. Biotic factors may play a crucial role in determining whether or not GEMs colonize a specific ecosystem since they interact with indigenous microbial populations.

Predictions of the colonization success of non-indigenous organisms focus on their biological characteristics [5] as well as on species-community interactions [21, 22]. Introductions may be more likely to succeed in communities with low species richness [6, 7, 21, 22]. Perturbations can reduce species richness and cause destruction of homeostatic mechanisms; under such conditions, introduced species often become dominant. Increased colonization success of novel organisms in such habitats may be due to the presence of vacant niches [21] and reduced intensity of predation and competition [7]. Colonization by GEMs may be favored in such habitats.

This study compared GEMs colonization success in ecologically perturbed and unperturbed aquatic systems and attempted to elucidate possible reasons for longer sur-

vival of GEMs in systems that underwent stress. Genetically-engineered *Erwinia carotovora*, which was used as a model, was constructed from the soft-rot-causing wildtype. Pathogenically disarmed *E. carotovora* may be useful for suppressing soft rot of potato seed pieces. This GEM was chosen for this study for its potential ecological and economic importance as a biological control agent.

Wildtype erwiniae are often present in surface waters [12, 14]; however, both wildtype and genetically-engineered *E. carotovora* decline below the level of detection after introduction into unperturbed aquatic microcosms [20].

Microorganisms can disperse away from the site of their introduction. After the field release of genetically-engineered *E. carotovora*, some viable cells will eventually disseminate to a non-target environment such as water. The GEMs may reach aquatic systems that are perturbed as a result of natural or anthropogenic stresses (e.g., short-term, acute stress from the release of an easily degradable or heated effluent). Such a perturbation results in elimination of indigenous organisms and simplifies the system, leaving it vulnerable to colonizers. We assessed the potential for establishment of GEM populations in unperturbed and perturbed systems using aquatic microcosms.

The second part of the experiment investigated the recovery of indigenous bacteria following thermal stress. Recovery of indigenous bacterial populations and their biological activities may be delayed as a result of competitive exclusion by GEMs that are already present in the perturbed system. Colonization of bacterial populations that utilize the same substrates as the genetically-engineered *E. carotovora* are most likely to be inhibited. Therefore, following the stress, the effect of GEMs on recolonization by total, proteolytic, cellulolytic, and pectolytic bacteria was examined.

Materials and Methods

Bacterial Strain

The GEM used in this experiment was constructed from *Erwinia carotovora* subsp. *carotovora* strain EC14, a pathogenic bacterium that causes soft rot of plant tissues [18]. Wildtype *E. carotovora* produces a battery of enzymes (pectic enzymes, cellulases, phospholipases, and proteases) responsible for the breakdown of components of plant tissue [13]. The GEM was constructed by disarming *in vitro* an extracellular pectate lyase by deletion mutation, inserting part from Tn*903* conferring kanamycin resistance, and chromosomally inserting part of the engineered plasmid in the pathogen's chromosome [20]. The genetically-engineered strain exhibits a 30% reduction in pathogenicity compared to the wildtype pathogen. To aid in isolation of the GEM, we used a spontaneous mutant resistant to 150 mg/l rifampicin, designated L-864.

Microcosm Preparation and Sampling

Each microcosm was contained in an 850-ml glass jar. Periphyton and water for microcosms were collected at Pandapas Pond, Virginia. Periphyton was obtained by rinsing it from the leaf litter. Dry weight of the periphyton-water suspension was 679 mg/l; ash-free dry weight was 71 mg/l. Six microcosms were sealed and placed in an incubator at 48°C ("perturbation") for 48 hours. The other six microcosms were kept in a 20°C incubator. Mild thermal perturbation was chosen because it presumably did not have a direct negative or positive effect on the GEMs introduced after the stress had passed. The microcosms were cooled to 20°C for 4 hours. Three perturbed and three unperturbed microcosms were treated with addition of 10^6 CFU of GEMs/ml. Noninoculated perturbed and unperturbed microcosms were used as controls. Bacterial culture was prepared, and cells were inoculated as described in Scanferlato *et al.* [20]. Microcosms were incubated at 20°C on a 12 hour light cycle. A flow-through system delivered filter-sterilized air, which mixed the water column. Prior to the sampling, the contents of each microcosm were gently mixed with a sterile pipette. A 1-ml or 0.1-ml sample was removed, serially diluted in sterile distilled water, and plated on appropriate medium.

Media

Genetically-engineered *E. carotovora* were enumerated using plate count agar (PCA; Difco, Detroit, MI) fortified with 40 μg/ml kanamycin monosulfate and 150 μg/ml rifampicin. Total bacteria were enumerated on 1/10 strength PCA (2.35 g dehydrated PCA plus 13.5 g Difco Bactoagar), because most colonies were recovered at this dilution. Numbers of proteolytic bacteria were determined by inoculation on Colwell YE medium [19]. Numbers of cellulolytic bacteria were estimated from Riviere agar [19]. Pectolytic bacteria were isolated on the basal medium for crystal violet pectate medium [9] lacking the additions of crystal violet and sodium lauryl sulfate, which are selective agents inhibiting growth of some pectolytic bacteria. Plates with *E. carotovora* were incubated at 30°C for 48 hours. The other plates were incubated at 20°C, for 48 hours (proteolytic bacteria), 96 hours (total and pectolytic bacteria), or 192 hours (cellulolytic bacteria). Counts were expressed as CFU/ml of water.

Indigenous proteolytic, cellulolytic, and pectolytic bacteria (competitors of genetically-engineered *E. carotovora* for the same substrates) were monitored during the experiment, since the differences in their densities in perturbed and unperturbed microcosms might explain different survival lengths of GEMs. Since *E. carotovora* grows on selective media for the functional groups examined, the densities of total, proteolytic, cellulolytic, and pectolytic bacteria could not be distinguished from GEMs density until the GEMs declined below the density of specific groups.

Densities of total, proteolytic, cellulolytic, and pectolytic bacteria in perturbed microcosms inoculated with GEMs were compared with densities of the same bacterial groups in noninoculated perturbed microcosms to determine potential differences in recovery rates after the temperature stress. Two parallel processes determined bacterial densities in perturbed microcosms inoculated with GEMs -- decline of the GEMs populations and the increase of population densities of survivors of thermal stress. Consequently, we could not distinguish the dynamics of indigenous bacteria during the first 5 days after GEMs inoculation, since they were present in densities lower than the GEMs.

Biomass

Water-detritus suspension (10 ml) was filtered through an ashed and preweighed glass fiber filter (GFF/A). Dry weight (DW) was determined after drying the filter at 105°C for 1 hour. Ash-free dry weight (AFDW) is the difference between DW and weight determined after ashing the filter at 550°C [3].

Biological Activity

We determined the effect of GEMs addititon on biological activities in perturbed and unperturbed microcosms. Biological activity was quantified by measuring respiratory electron transport system (ETS) activity of the respiratory chain in water samples using an artificial acceptor of electrons 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). Previous studies show that ETS activity correlates with oxygen consumption rates [17]. This method has been accepted for measuring biological activities in different ecosystems. A water-detritus suspension (10 ml) from each microcosm was filtered through a GFF/A, and the method of Owens and King [17] was used.

Experimental Design and Statistical Analysis

One-way analysis of variance (ANOVA) and the least significant difference (LSD) test were used to determine statistical significance among different treatments.

Results

GEMs Persistence

Genetically-engineered *E. carotovora* were detected for 35 days after inoculation into perturbed aquatic microcosms. They were not detectectable after only 5 days in the un-perturbed microcosms (Fig. 1).

Community Change

Perturbation significantly decreased the densities of bacterial populations. Density of total bacteria in unperturbed microcosms was 490 x 10^3 cells/ml water and 6.2 x 10^3 cells/ml in perturbed microcosms immediatelly after the thermal stress. Densities of cellulolytic bacteria decreased from 270 x 10^3 cells/ml to 4.3 x 10^3 cells/ml; densities of

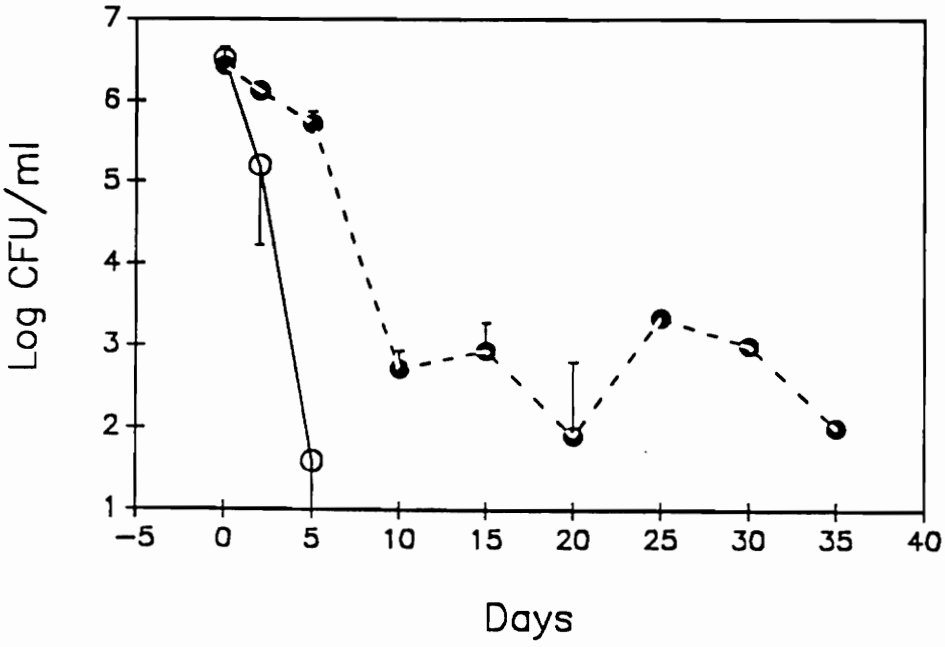

Fig. 1. Survival of genetically-engineered *E. carotovora* in thermally unperturbed (○) and perturbed (●) microcosms. Perturbed aquatic microcosms were incubated at 48°C for 48 hours; unperturbed microcosms were incubated at 20°C. Error bars represent one standard deviation and are included within the symbol when not shown.

proteolytic bacteria decreased from 72 x 10^3 cells/ml to 7.4 x 10^3 cells/ml. Perturbation did not significantly reduce densities of pectolytic bacteria (61 cells/ml, $P > 0.05$).

Total bacteria (Fig. 2A) increased in perturbed inoculated microcosms to higher densities than in unperturbed inoculated microcosms 10 days after the treatment with GEMs ($P = 0.054$) and remained significantly higher for the duration of the experiment ($P < 0.05$). Densities of proteolytic bacteria (Fig. 2B) were not significantly different in perturbed and unperturbed GEM-inoculated microcosms at days 10, 15, and 30 ($P > 0.05$), but were significantly higher in perturbed microcosms at days 20 and 25 ($P < 0.05$). Cellulolytic bacteria (Fig. 2C) were present in significantly higher densities in perturbed inoculated microcosms 10 to 30 days after treatment with GEMs ($P < 0.05$). Densities of pectolytic bacteria in perturbed inoculated microcosms (Fig. 2D) were significantly higher than in unperturbed inoculated microcosms from day 5 to day 30, due to the presence of GEMs ($P < 0.05$).

Rotifers, nematods, and cladocerans were present during the experiment in unperturbed microcosms, but were eliminated from temperature-stressed microcosms. Nineteen protozoan species were found at the end of the experiment in unperturbed microcosms, representing 14 genera (*Vorticella, Valhkampfia, Pleuromonas, Monas, Peranema, Bodo, Cercomonas, Chlamydomonas, Filamoeba, Cyclidium, Chromulina, Phacus, Euglena,* and *Notoselenus*; B. Niederlehner, personal communication). In perturbed microcosms, dense populations of colorless bactivorous nanoflagellates *(Monas, Pleuromonas,* and *Cercomonas)* developed after the stress. Until the end of the experiment, these were the only organisms other than bacteria observed in perturbed microcosms.

Ash-free dry weight/dry weight ratio (AFDW/DW) was not significantly different ($P > 0.05$) in perturbed and unperturbed microcosms in the beginning of the experiment ($10.38 \pm 0.99\%$, mean $\pm$ 1 SD). However, the AFDW/DW ratio was significantly higher

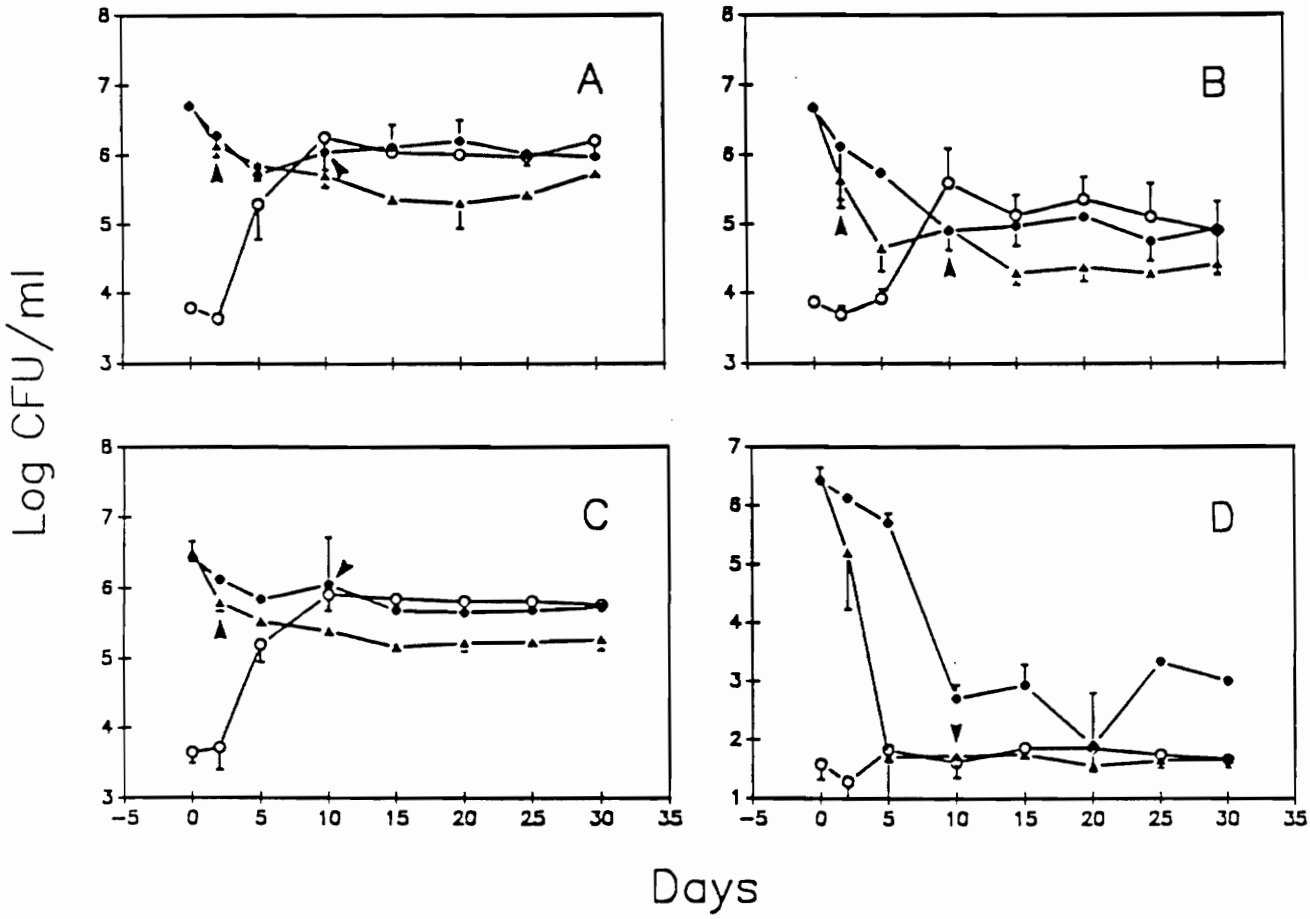

Fig. 2. Density of total bacteria (A), proteolytic bacteria (B), cellulolytic bacteria (C), and pectolytic bacteria (D) in perturbed noninoculated microcosms (○), unperturbed microcosms inoculated with GEMs (▲), and perturbed microcosms inoculated with GEMs (●). Microcosms were perturbed thermally as described in Fig. 1. Arrows (▼) indicate the points at which densities of protein-, cellulose-, and pectate-degrading *E. carotovora* declined below population densities of indigenous proteolytic, cellulolytic, or pectolytic bacteria. Error bars represent one standard deviation and are included within the symbols when not shown.

($P < 0.05$) at the end of the experiment (day 30) in unperturbed microcosms (10.57 $\pm$ 0.63%) than in perturbed microcosms (9.04 $\pm$ 0.35%), indicating a decrease in organic matter content in perturbed microcosms.

Respiratory ETS activity was depressed to 92 $\pm$ 61 ug/g AFDW/hour in perturbed microcosms, compared to 689 $\pm$ 169 ug/g AFDW/hour in unperturbed noninoculated microcosms.

GEMs Effect on Recovery

Antagonism due to competition may occur between GEMs and indigenous bacteria. Since the niches may already be occupied by protein-, cellulose-, and pectate-degrading GEM, this might retard or prevent colonization by the indigenous microbial populations.

Total bacteria reached their maximal density 10 days after perturbation (Fig. 2A). At day 5 and after, the densities of total bacteria in perturbed GEM-inoculated microcosms were not significantly different from their densities in perturbed noninoculated microcosms ($P > 0.05$). Densities of indigenous proteolytic bacteria were significantly lower 10 days after perturbation in inoculated microcosms than in noninoculated ones ($P < 0.05$), but not afterwards (Fig. 2B). This might indicate that indigenous proteolytic bacteria recover more slowly from stress when GEMs are present; however, final densities in perturbed GEM-inoculated microcosms and perturbed noninoculated microcosms were similar. Recovery of cellulolytic bacteria in perturbed microcosms (Fig. 2C) did not appear to be affected by GEMs addition. Differences in densities of cellulolytic bacteria in perturbed noninoculated and inoculated microcosms were not significant after day 5 ($P > 0.05$). Pectolytic GEMs (Fig. 1, 2D) remained in perturbed microcosms throughout the experiment in significantly higher densities than pectolytic bacteria in perturbed noninoculated microcosms ($P < 0.05$).

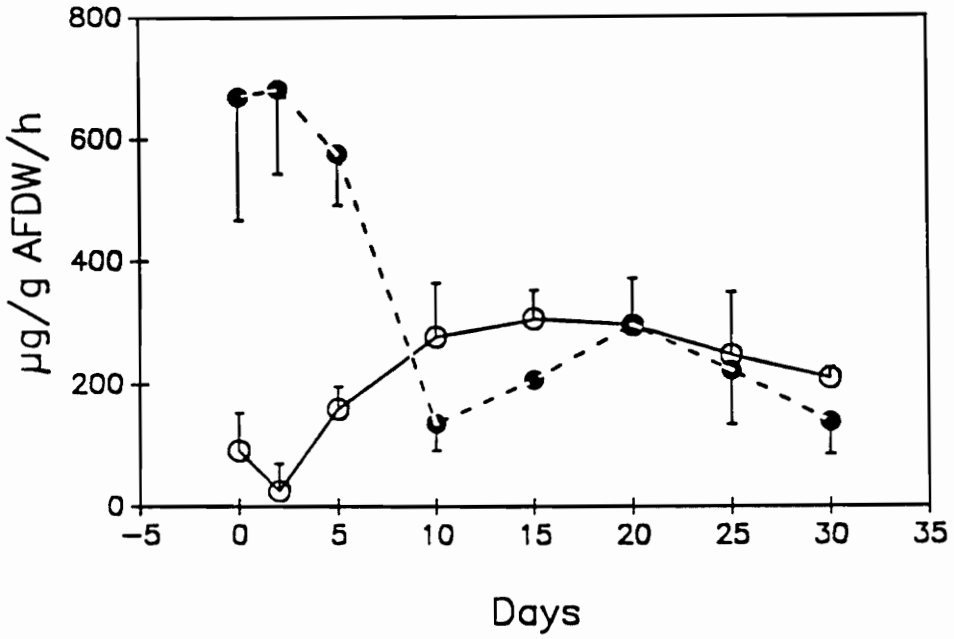

Fig. 3. Respiratory electron transport system activity in thermally perturbed (48°C for 48 hours) uninoculated microcosms (○) and in perturbed microcosms inoculated with genetically-engineered *E. carotovora* (●). Error bars represent one standard deviation and are included within the symbols when not shown.

Addition of GEMs caused an increase of respiratory ETS activity in perturbed microcosms to the levels significantly higher than noninoculated perturbed microcosms (Fig. 3). Activity in perturbed noninoculated microcosms steadily increased, and at day 10 reached a level similar to that in perturbed microcosms treated with GEMs ($P > 0.05$). This suggests that the increase in the respiratory ETS activity due to GEMs addition was transitory. Recovery of the respiratory ETS activity of indigenous populations was not supressed due to GEMs presence.

Discussion

Because of the potential for GEMs to spread to localities other than the site of their release, it is essential to determine their survival under different conditions. Survival of GEMs should be tested carefully in the environments that are most likely to become colonized. According to the biotic resistance hypothesis and the equilibrium theory of island biogeography, immigrations are more numerous and more likely to succeed where few species are present [22]. Previous experience with introduced organisms shows that invasions are likely to be successful in perturbed systems [21, 22].

Community structure and function in our study underwent a drastic change due to thermal perturbation. Densities of total bacteria and specific functional groups decreased 1 to 2 orders of magnitude. The protozoan assemblage was reduced to three species of colorless flagellates, and all other eukaryotes were eliminated. Biological activity of the community was significantly depressed after perturbation.

Introduced GEMs declined rapidly in unperturbed microcosms. Similar decline of genetically-engineered *E. carotovora* was observed in previous experiments after introduction to aquatic microcosms [20]. The GEMs persisted considerably longer in the microcosms perturbed by thermal stress. Perturbation should have left a number of niches vacant and available for colonization by the GEMs. Studies conducted in steri-

lized systems suggest that the lack of competitive and predatory pressure is responsible for longer survival of bacteria in sterile environments [10, 15]. We examined nutrient availability, competition for nutrients, and predation as the most important factors that could affect bacterial levels.

The rapid decline of genetically-engineered *E. carotovora* in unperturbed microcosms may have resulted from resource limitations. In thermally-perturbed microcosms, killed organisms were available as a source of nutrients for bacterial degradation. Significantly lower AFDW/DW ratios in perturbed microcosms at the end of the experiment also reflect the decrease of organic matter content, probably because of the bacterial degradation of available nutrients. Increased densities of proteolytic and cellulolytic bacteria in perturbed microcosms indicate an increased availability of nutrients, including proteins and cellulose as substrates for *E. carotovora*. Growth of bacteria to densities greater than in the control was observed by Bloem and colleagues [4] after the addition of the cell extracts into lake water. Nutrient-rich environments may be more suitable for *E. carotovora*, which is a plant pathogen, than is an oligotrophic water environment of unperturbed microcosms.

Perturbation caused a temporary reduction in bacterial density. Bacteria which used the same classes of nutrients (pectate, proteins, cellulose) as genetically-engineered *E. carotovora* recovered quickly. These potential competitors reached the same or higher densities in perturbed than in unperturbed microcosms by day 10. However, GEMs persisted in perturbed microcosms 10 to 25 days afterwards, despite the greater density of competitors. Other factors, including abundance of nutrients and decreased predatory pressure, may have mitigated any detrimental effects of increased competition on GEMs survival. In experiments that use irradiation or autoclaving as a means of sterilization, increase of available nutrients resulting from dead cells may be a primary reason for enhanced persistence of non-oligotrophic bacteria, rather than decreased competition.

Grazing can be a major cause of bacterial mortality in aquatic systems [4, 15]. Longevity of the bacterium *Yersinia enterolytica* was significantly extended when a eukaryote inhibitor was added to the river water or river water was passed through a 0.8-um filter to remove eukaryotic predators [8]. Grazing by zooplankton and microplankton accounted for the loss of bacterial viability in untreated lake water compared to that in selectively filtered lake water [2]. Bacterial populations reached the stationary phase after exponential growth in the grazer-free control, but were rapidly grazed down in the presence of grazers [4]. Similarly, intensive grazing is a probable cause for the rapid decline of bacteria in the unperturbed microcosms in our experiment. Bacteria may coexist with predators if they are able to multiply and replace cells that were eliminated by predators [1]. Disappearance of GEMs from microcosms indicates that more cell were lost than were generated.

Since the potential carrying capacity of a system is rather constant, competitive exclusion by GEMs may be responsible for the failure of the indigenous community to recover. Lindow [16] found that ice-minus bacteria established on plant surfaces reduce the subsequent colonization of ice nucleation bacteria. In the present study, no difference was observed in the recovery of bacteria from any functional group (cellulolytic, pectolytic, and pectolytic) in microcosms treated with GEMs, compared with their recovery in uninoculated, perturbed microcosms. In the time frame we used, there was little evidence that pre-emptive inoculation with GEMs reduced the ability of the indigenous bacterial populations to recover from perturbation.

The fact that GEMs exhibited negative exponential decline and persisted at lower densities than indigenous groups, whereas indigenous bacteria grew to the stationary phase, suggests the unsuitability of the aquatic habitat for genetically-engineered *E. carotovora.* It appears that a certain bacterial density can be maintained by the system, regardless of the presence of GEMs. Vacant niches and nutrient availability favored the

survival of GEMs after perturbation, but they declined as soon as the nutrients were sequestered by the indigenous populations. The possibility of colonization of aquatic habitats, even of perturbed ones, by genetically-engineered *E. carotovora* seems, therefore, remote.

Acknowledgments. This study was supported by Sigma Xi Grant-in-Aid of Research. We are grateful to P. McCormick, D. Orvos, and B. Wallace for helpful suggestions, to B. Niederlehner for protozoan identifications, and to Darla Donald for preparing the manuscript for publication.

References

1. Alexander M (1981) Why microbial predators do not eliminate their prey and hosts. Annu Rev Microbiol 35:113-133
2. Amy PS, Hiatt HD (1989) Survival and detection of bacteria in an aquatic environment. Appl Environ Microbiol 55:788-793
3. APHA, AWWA, WPCF (1985) Biological examination of water. In: Franson MAH (ed) Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC, pp 1041-1215
4. Bloem J, Albert C, Bar-Gillissen MB, Berman T, Cappenberg T (1989) Nutrient cycling through phytoplankton, bacteria, and protozoa, in selectively filtered Lake Vechten water. J Plankton Res 11:119-131
5. Brock T (1985) Procaryotic population ecology. In: Halvorson HO, Pramer D, Rogul M (eds) Engineered organisms in the environment: Scientific issues. American Society for Microbiology, Washington, DC, pp 177-179
6. Brown JH, Gibson AC (1983) Biogeography. C. V. Mosby Co, St. Louis, MO
7. Cairns J Jr, Pratt JR (1986) Factors affecting the acceptance and rejection of genetically altered microorganisms by established natural aquatic communities. In:

Poston TM, Purdy R (eds) Aquatic toxicology and environmental fate: Ninth symposium, ASTM STP 921. American Society for Testing and Materials, Philadelphia, PA, pp 207-221

8. Chao WL, Ding RJ, Chen RS (1988) Survival of *Yersinia entorolitica* in the environment. Can J Microbiol 34:753-756
9. Cuppels D, Kelman A (1974) Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue. Phytopathology 64:468-475
10. de Boer HS (1982) Survival of phytipathogenic bacteria in soil. In: Mount MS, Lacy GH (eds) Phythogenic prokaryotes, vol.1. Academic Press Inc, New York, pp 88-108
11. Fiksel JR, Covello VT (1986) The suitability and aplicability of risk assessment methods for environmental application in biotechnology. In: Fiksel JR, Covello VT (eds) Biotechnology risk assessment: issues and methods for environmental introductions. Pergamon Press, New York, pp 1-34
12. Harrison MD, Franc GD, Maddox DA, Michaud JE, McCarter Zorner NJ (1987) Presence of *Erwinia carotovora* in surface water in North America. J Appl Bacteriol 62:565-570
13. Kotoujansky A (1987) Molecular genetics of pathogenesis by soft-rot erwinias. Ann Rev Phytopathol 25:405-430
14. Lacy GH, Lamb RC, Berg CM (1981) Iris soft rot caused by *Erwinia chrysanthemi*, assosiated with overhead irrigation and its control by chlorination. Vol 31 International Plant Propagators' Society Combined Proceedings for 1981 31:624-634
15. Liang LN, Sinclair JL, Mallory LM, Alexander M (1982) Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl Environ Microbiol 44:708-714
16. Lindow SE (1987) Competitive exclusion of epiphytic bacteria by ice- *Pseudomonas syringae* mutants. Appl Environ Microbiol 53:2520-2527

17. Owens TG, King FD (1975) The activity measurement of respiratory electron-transport-system activity in marine zooplankton. Marine Biol 30:27-36

18. Roberts DP, Berman PM, Allen C, Stromberg VK, Mount, MS (1986) *Erwinia carotovora*: Molecular cloning of a 3.4 kilobase DNA fragment mediating production of pectate lyases. Can J Plant Pathol 8:17-27

19. Rodina AG (1965) Methody vodnoi microbiologii. Translated 1972 as Methods in aquatic microbiology. Colwell R, Zambruski MS (eds), University Park Press, Baltimore, MD

20. Scanferlato VS, Orvos DR, Cairns J Jr, Lacy GH (1989) Genetically-engineered *Erwinia carotovora* in aquatic microcosms: Survival and effects on functional groups of indigenous bacteria. Appl Environ Microbiol 55:1477-1482

21. Sharples FE (1983) Spread of organisms with novel genotypes: Thoughts from an ecological perspective. Recomb DNA Tech Bull 6:43-56

22. Simberloff D (1985) Predicting ecological effects of novel organisms: Evidence from higher organisms. In: Halvorson HO, Pramer D, Rogul M (eds) Engineered organisms in the environment: Scientific issues. American Society for Microbiology, Washington, DC, pp 152-161

23. Stacey G (1985) The *Rhizobium* experience. In: Halvorson HO, Pramer D, Rogul M (eds) Engineered organisms in the environment: Scientific issues. American Society for Microbiology, Washington, DC, pp 109-121

24. Strauss HS, Hattis D, Page G, Harrison K, Vogel S, Caldarf C (1984) Genetically-engineered microorganisms: II. Survival, multiplication and genetic transfer. Recomb DNA Tech Bull 9:69-88

Chapter 4

Competition, survival, and ecological effects of wildtype and genetically-engineered *Erwinia carotovora* strains in aquatic microcosms *

1. SUMMARY

Survival, effect on specific groups of indigenous bacteria, and intraspecific competition of genetically-engineered and wildtype *Erwinia carotovora* were followed in aquatic microcosms. In water or in sediment, densities of both strains declined at the same rate and fell below a level of detection. The effect of engineered and wildtype *E. carotovora* on densities of total bacteria, Actinomycetes, and *Pseudomonas* spp. was not significantly different. In a competition experiment, wildtype was not displaced by the engineered

* This chapter has been submitted to FEMS Microbiology Ecology with the authors V. S. Scanferlato, G. H. Lacy, and J. Cairns, Jr.

E. carotovora. **Treatment with engineered bacteria did not change biomass values (ash-free dry weight) of the receiving community, but caused a transitory increase in its metabolic activity. The inability of genetically-engineered *E. carotovora* to persist, displace resident species, and affect the metabolic activity of a community indicates the low risk of adverse ecological consequences in aquatic ecosystems.**

2. INTRODUCTION

Field releases of genetically-engineered microorganisms (GEMs) have been limited to small-scale trials, mainly because of the lack of knowledge about potential ecological consequences. Problems that complicate the assessment of environmental risk are the GEMs ability to exchange engineered genes [1], exist in a viable, but non-culturable stage [2], disperse from the site of release [3], persist in densities below the detection limit [4,5], and increase in numbers at suitable habitats [6]. Since different factors add to the uncertainty about GEMs potential environmental impact, release of GEMs should be examined on a case-by-case basis [7].

GEMs introduced into an ecosystem interact with resident microbial populations [8]. Intraspecific competition between the engineered and indigenous wildtype strain may favor the establishment of the former, if it is ecologically more successful in a specific environment [9]. Furthermore, intercompetitive interactions may cause changes in natural microbial communities. Displacements of bacterial populations, which play a role in mineralization of organic matter, are considered particularly important in aquatic ecosystems, in which the largest amount of biomass and organic carbon exists in the form of detritus [10]. Disruption of mineralization processes may lead to the changes in the productivity of aquatic ecosystems [7,11].

Since GEMs may disseminate from the site of their release, it is important to examine survival and impact of GEMs under different environmental conditions. Experiments involving the first step in risk assesment are usually performed in microcosms as surrogates of natural ecosystems. Such an approach eliminates risks associated with field tests that may result from the absence of reliable recall mechanisms for GEMs [12].

Genetically-engineered *Erwinia carotovora* subsp. *carotovora* L-833 was used as a model in these studies because of its potential ecological and economic importance as a biological control agent. The GEM was constructed from the wildtype pathogen, the causal agent of soft rot in plant tissues [13]. Wildtype *E. carotovora* is universally present in aquatic ecosystems [14,15]. Concentrations of erwiniae higher than 10^7 cells/ml have been found in surface waters containing rotting plant material [23].

In order to determine the potential environmental risk that may result from the presence of genetically-engineered *E. carotovora* in aquatic systems, we determined (a) the ability of the genetically-engineered strain to survive in the water and sediment of aquatic microcosms, (b) the effect of GEMs on specific groups of indigenous bacteria, (c) the result of intraspecific competition between the GEM and wildtype, and (d) the effect of GEMs on biomass and metabolic activity of the receiving community.

3. MATERIALS AND METHODS

3.1. Bacterial strains

Erwinia carotovora subsp. *carotovora* produces a battery of enzymes that are responsible for the breakdown of plant tissue components [16]. Genetically-engineered *E. carotovora* strain L-833 was constructed from wildtype *E. carotovora* strain EC14 by chromosomal insertion of a DNA fragment containing a disarmed pectate lyase gene, marked with a 1.4 kb DNA fragment from Tn*903* conferring kanamycin resistance

[13,17]. Spontaneous rifampicin mutants (150 ug/ml) of EC14 and L-833 were designated L-863 and L-864, respectively. The wildtype rifampicin mutant L-863 and the genetically-engineered strain L-833 were used in the competition study. Survival in the absence of the other *E. carotovora* strain was assessed using the wildtype L-863 and the genetically-engineered L-864 strain. Cultures of genetically-engineered and wildtype *E. carotovora* were grown in nutrient broth to a density of approximately 10^9 CFU/ml, estimated turbidiometrically at 550 nm. Cells were harvested by centrifugation at 10,000 x *g*, and washed twice in sterile distilled water.

3.2. Microcosms

GEM survival, intraspecific competition, and effect on indigenous bacteria were studied in microcosms, which were constructed in 850-ml glass jars using 300 ml of sediment and 550 ml of water [13]. Microcosms were inoculated into the water phase with 10^7 CFU/ml of genetically-engineered and/or wildtype *E. carotovora* and incubated at 20°C on a 12-h light cycle. The water column was sampled at approximately 2 cm beneath the surface, and sediment samples were obtained by inserting a serological plastic pipet with its conical tip removed through the depth of the sediment. The bottom 1.0 ml of the sediment core was used.

The effect of GEMs on biomass and metabolic activity was studied in microcosms containing 850 ml of water and periphyton. Dry weight of such a suspension was 679 mg/l. Each microcosm received 10^7 CFU/ml of genetically-engineered *E. carotovora*. Uninoculated microcosms served as controls. Prior to sampling, water-periphyton suspension was mixed with a sterile pipet.

3.3. Media

Samples were diluted serially and plated on appropriate media. Genetically-engineered *E. carotovora* strain L-833 was enumerated using plate count agar (PCA; Difco, Detroit, MI) fortified with 40 ug/ml kanamycin monosulfate. Densities of genetically-engineered strain L-864 were determined by plating on PCA amended with 40 ug/ml kanamycin monosulfate and 150 ug/ml rifampicin. Wildtype strain L-863 was enumerated on PCA amended with 150 ug/ml rifampicin. Total bacteria were enumerated on 1/10 PCA (2.35 g/l PCA with 13.5 g/l Difco Bactoagar), because most colonies were recovered at this dilution of the medium. Actinomycetes and pseudomonads were estimated on Actinomycetes Isolation Agar (Difco) and Pseudomonas Isolation Agar (Difco), respectively. Densities of *E. carotovora* and pseudomonads were estimated from plates incubated at 30°C for 48 h. Densities of total bacteria and Actinomycetes were estimated from plates incubated at 20°C for 96 h. Counts were expressed as CFU/ml of water or sediment.

3.4. Effect on biomass

Ash-free dry weight (AFDW) was used in this study as an estimate of biomass and organic matter content. Effect of GEMs on community biomass was determined by passing 10 ml of water through an ashed and pre-weighed glass fiber filter (GFF/A). The filter was dried 24 h at 105°C, weighed, re-ashed 1 h at 550°C, and weighed again [18]. The difference between the two weights represents AFDW.

3.5. Effect on metabolic activity

Impact of GEMs on metabolic activity of the receiving community was determined by measuring respiratory electron transport system activity (ETS) of the respiratory chain, using 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) as

an artificial acceptor of electrons. The ETS activity of a 10-ml sample filtered through a GFF/A was measured as described by Owens and King [19].

3.6. Experimental design and statistical analysis

Log transformed values of bacterial densities were used in the statistical analysis. A two-sample Student's *t* test was used to evaluate statistical significance on individual sampling days between (a) densities of the wildtype and genetically-engineered *E. carotovora* in survival and competition studies, (b) densities of specific bacterial groups in microcosms inoculated with the wildtype and genetically-engineered strain, and (c) biomasses and respiratory ETS activities in microcosms inoculated with genetically-engineered *E. carotovora* and in noninoculated control microcosms.

4. RESULTS

4.1. Survival

In the first study, genetically-engineered L-864 and wildtype L-863 *E. carotovora* were inoculated into separate microcosms. Their populations declined in the water and sediment of aquatic microcosms (Fig. 1). On individual sampling days, the densities of the two strains in water or in sediment were not significantly different ($p > 0.05$). The average decline rates of *E. carotovora* for the first 12 days after inoculation were 0.36 log units/day (water) and 0.13 log units/day (sediment). Neither the wildtype nor genetically-engineered strain were detected by plating 24 days after inoculation (detection limit was 10 cells/ml water and 100 cells/ml sediment).

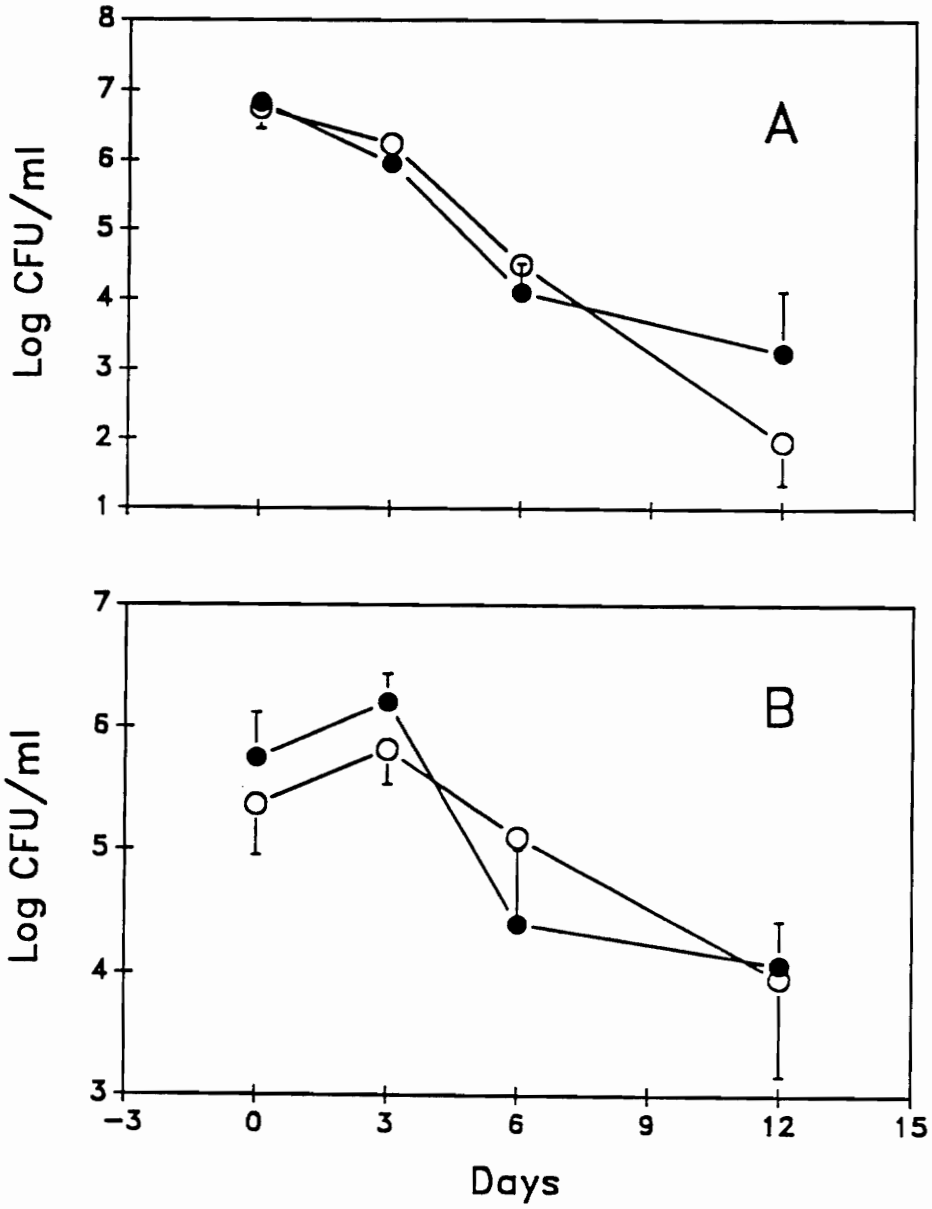

Fig. 1. Survival of ○, genetically-engineered (L-864) and ●, wildtype (L-863) strains of *E. carotovora* in the water (A) and sediment (B) of aquatic microcosms. Initial inoculation levels were 10^7 CFU/ml water. Separate microcosms were inoculated with single strain of *E. carotovora* Error bars represent one standard deviation unit and are included within the symbol when not shown.

4.2. Competition

The second study assessed competition between the genetically-engineered L-833 and the wildtype L-863 strains. Both *E. carotovora* strains were simultaneously inoculated into aquatic microcosms. The two strains declined at a rate of 0.5 log units/day in water and at a rate of 0.24 log units/day in sediment (Fig. 2). Fifteen days after inoculation, neither strain could be detected in the water or sediment. Initial inoculum densities of the wildtype and genetically-engineered strain were not significantly different ($p > 0.05$). Likewise, the subsequent sampling of the water and sediment showed that densities of the two strains were not significantly different at any point of time afterwards ($p > 0.05$).

4.3. Effect on indigenous bacterial groups

The effects of genetically-engineered and wildtype strains on densities of indigenous bacteria are shown in Figure 3. The total bacterial density was not significantly different in microcosms inoculated with GEMs compared to microcosms treated with the wildtype strain ($p > 0.05$). An increase in total density was observed in water and sediment after inoculation, due to *E. carotovora.* Similarly, there were no significant differences in densities of Actinomycetes in microcosms inoculated with the GEM and in microcosms inoculated with the wildtype ($p > 0.05$). The increase in Actinomycetes density was observed in water and sediment after addition of the genetically-engineered or wildtype strains into aquatic microcosms. The effect of genetically-engineered *E. carotovora* on densities of *Pseudomonas* spp. was not significantly different from the effect of the wildtype on the same bacterial group ($p > 0.05$).

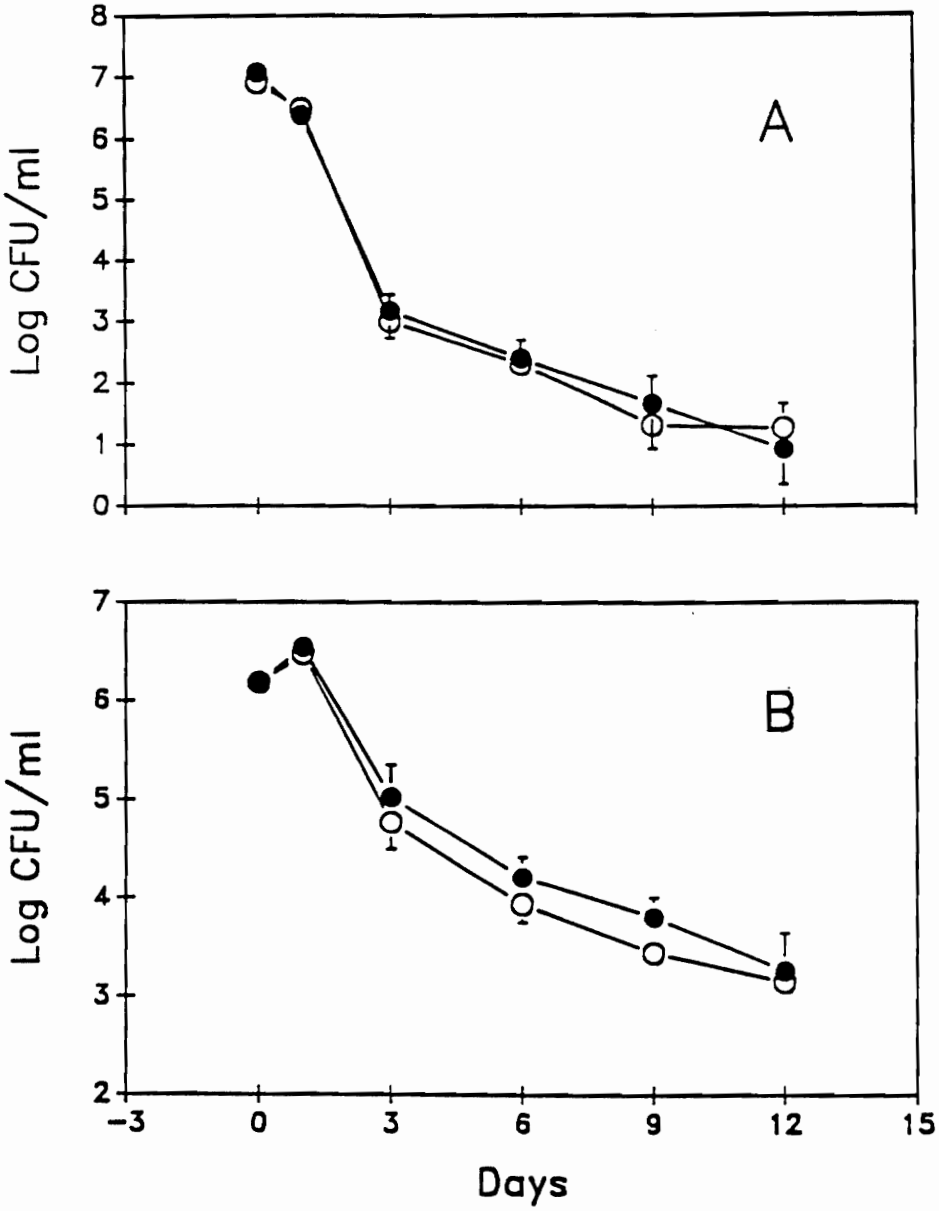

Fig. 2. Competitive survival of ○, genetically-engineered (L-833) and ●, wildtype (L-863) strains of *E. carotovora* in water (A) and sediment (B). Microcosms were inoculated simultaneously in densities of 10^7 CFU/ml water. Error bars represent one standard deviation unit and are included within the symbol when not shown.

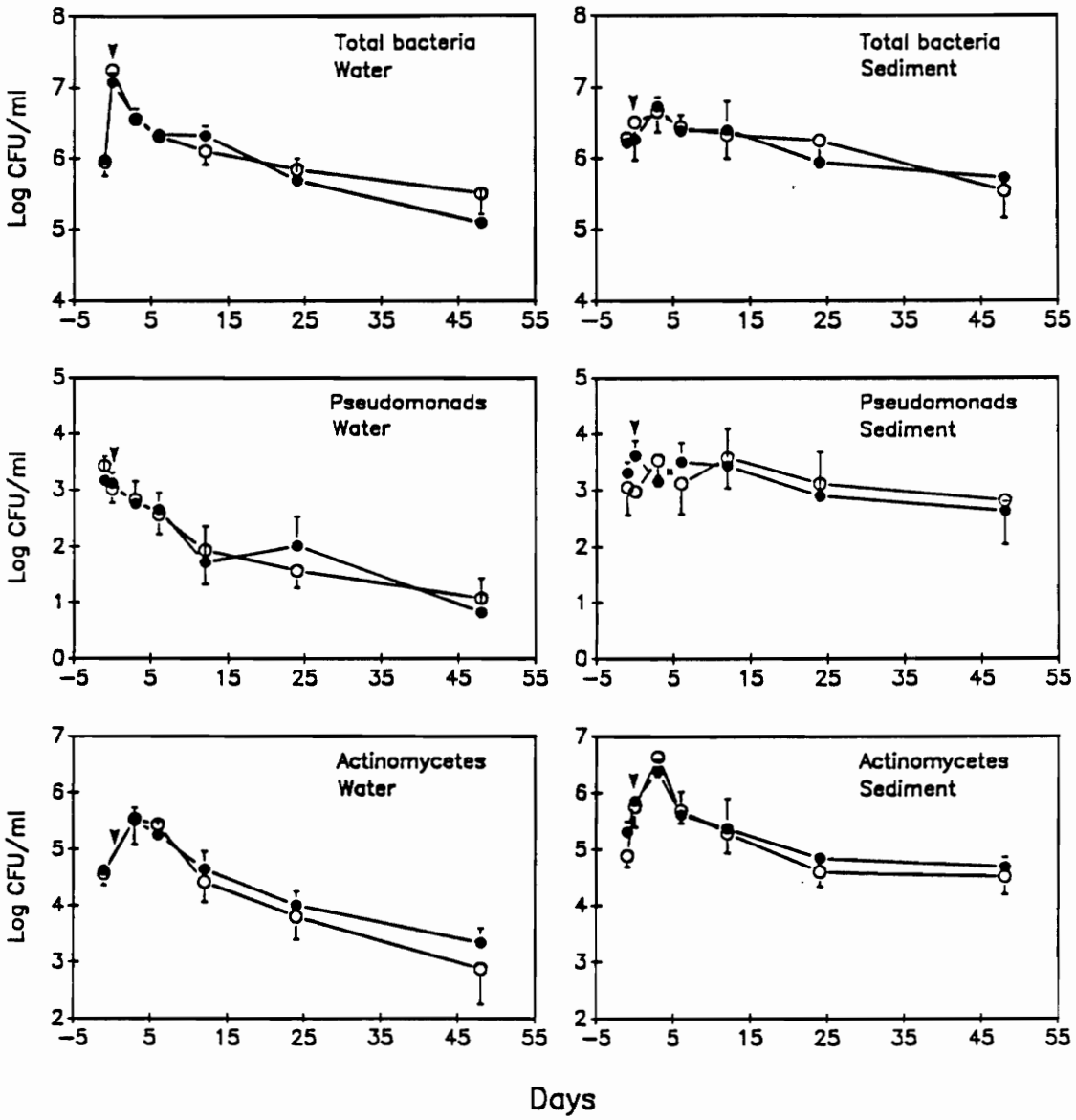

Fig. 3. Effect of *E. carotovora* on densities of total bacteria, pseudomonads, and Actinomycetes in water and sediment. ○, densities in microcosms inoculated with genetically-engineered strain (L-834); ●, densities in microcosms inoculated with the wildtype strain (L-863). Densities of a specific bacterial group one day before the inoculation with *E. carotovora* are indicated by arrows. Error bars represent one standard deviation unit and are included within the symbol when not shown.

4.4. Effect on biomass

Addition of GEMs to aquatic microcosms did not cause a change in biomass content (AFDW) of the system (Fig. 4). The biomass of the receiving community was 71 ± 11 ug AFDW/ml of water (mean ± standard deviation). Inoculation of 10^7 *E. carotovora* cells/ml was responsible for 2.2 μg AFDW/ml of water and did not cause a significant increase in AFDW ($p > 0.05$).

4.5. Effect on metabolic activity

Respiratory ETS activity was significantly higher for 5 days in microcosms inoculated with GEMs than in the uninoculated controls ($p < 0.05$, Fig. 5). The metabolic activity decreased as the populations of GEMs declined. After the initial period, respiratory ETS activity reached a level that was not significantly different in inoculated and uninoculated microcosms ($p > 0.05$).

5. DISCUSSION

Prior to the planned introduction of genetically-engineered microorganisms into the environment, potential ecological consequences should be considered. To assess the risk of release of genetically-engineered *E. carotovora*, it was important to determine their ability to persist and colonize, affect resident organisms, and change the functioning of the system.

Decline and disappearance of genetically-engineered and wildtype *E. carotovora* from the water column and sediment were observed after their inoculation into aquatic microcosms. This confirmed the results of previous experiments [13]. An initial rapid and a subsequent slow decrease of density was characteristic for both *E. carotovora*

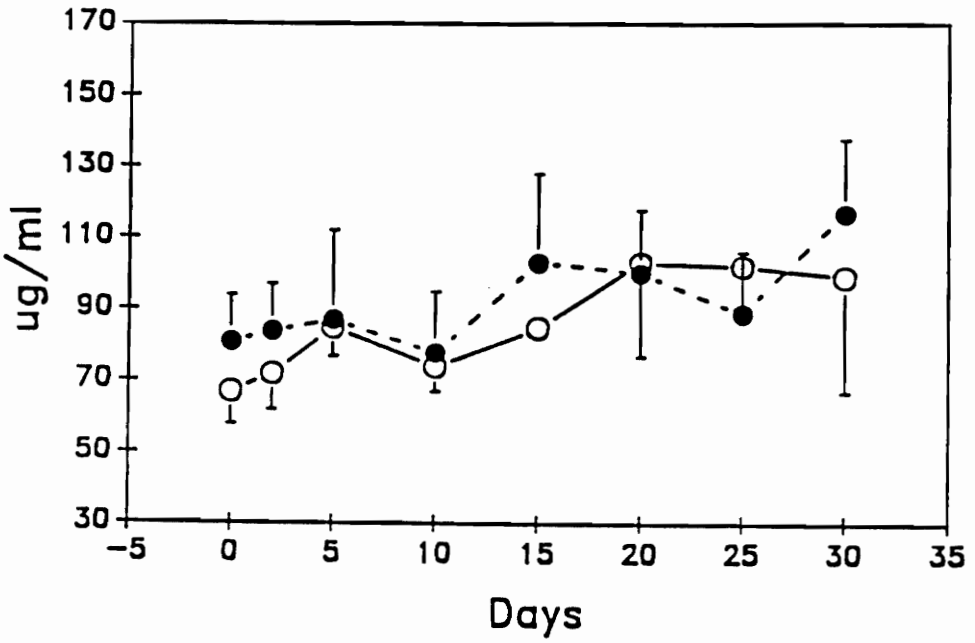

Fig. 4. Biomass (ash-free dry weight) in microcosms inoculated with genetically-engineered *E. carotovora* L-864 (●) and in noninoculated control microcosms (○). Error bars represent one standard deviation unit and are included within the symbol when not shown.

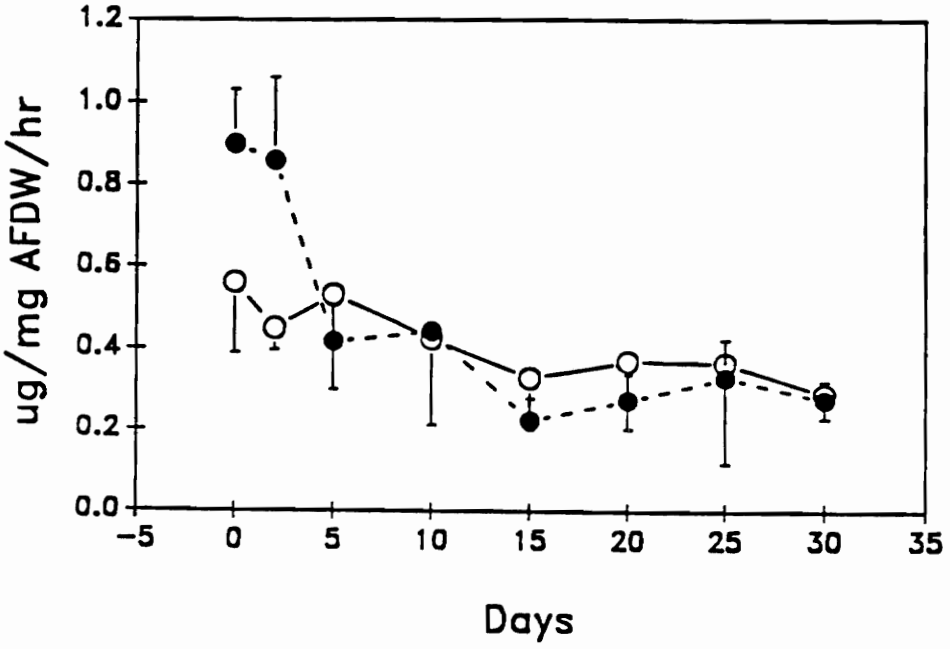

Fig. 5. Respiratory electron transport system activity in microcosms inoculated with genetically-engineered *E. carotovora* L-864 (●) and in noninoculated control microcosms (○). Error bars represent one standard deviation unit and are included within the symbol when not shown.

strains. Similar decline curves were observed in the competition experiment, in which the densities of simultaneously introduced wildtype and genetically-engineered *E. carotovora* populations decreased at a same rate. A low level of nutrients and predation were probably most responsible for elimination of the genetically-engineered and wildtype *E. carotovora* from the aquatic system [20,21]. The genetic alteration apparently did not affect the fitness and competitiveness of the engineered compared to the wildtype strain in water and in sediment. The presence of plant tissue may be expected to accentuate any differences between the two populations. However, a separate study showed that genetically-engineered *E. carotovora* increase in their density in potato tuber tissue at the same rate as the wildtype [22].

Large densities of wildtype erwiniae that occasionally appear in natural surface waters originate from terrestrial environments. Bacterial cells are introduced with rotting plant tissue into water [23]. However, it is doubtful if the external sources can account for the continuous presence of *E. carotovora* in surface waters [15]. There is a possibility that niches exist in natural aquatic systems where the wildtype can survive or colonize, in streambed sediments [24], or perhaps as pathogens or epiphytes on aquatic plants. Because of the similar physiological and ecological characteristics of the genetically-engineered and wildtype *E. carotovora*, such habitats may be suitable for the persistence of both strains.

After the GEMs release, resident microbial communities will first respond to potential perturbation [25]. We monitored changes in densities of total bacteria and densities of two bacterial groups involved in mineralization of organic matter -- Actinomycetes and *Pseudomonas* spp. In our experiments, the GEM did not displace these bacterial groups. The introduction of genetically-engineered bacteria did not induce microbial change in the water or sediment in a manner different from that of the wildtype.

Biomass and metabolic activity were monitored after inoculation of GEMs into aquatic microcosms. Changes in biomass (AFDW) were not detected, but significant increase in metabolic activity of the system followed the GEMs inoculation. The absence of noticeable increase in biomass can be explained by the fact that 10^7 cells added to each milliliter represented addition of only 2.2 μg AFDW/ml. That amount is negligible when compared to AFDW content in noninoculated control microcosms (71 μg AFDW/ml). On the other hand, actively respiring *E. carotovora* cells caused a significant increase in respiratory ETS activity when added to microcosms. The activity decreased as the population density decreased. These results support the finding of Martinez et al. [26] that inactivity of metabolic enzymes involved in ETS reactions is associated with culturability losses and cell lysis. Significant increase of respiratory ETS activity in our study occurred after the inoculation of a small amount of biomass in the form of bacterial cells. This indicates that the large amount of microcosm biomass existed in the form of dead organic matter, which did not contribute to the respiration of the system.

The differences between genomes of the genetically-engineered and wildtype strain are small; consequently, the genetically-engineered strain had virtually unmodified ecological properties and behaved in the water and sediment in a similar way to its wildtype parent.

Both genetically-engineered and wildtype *E. carotovora* declined after introduction into the microcosm. The GEMs did not outcompete the wildtype strain, displace the indigenous bacteria, or have a long-lasting effect on the biomass or metabolic activity of the aquatic community. This evidence suggests that the risk for genetically-engineered *E. carotovora* to cause adverse ecological effects in aquatic ecosystems is remote. However, we recommend that uncertainties about possible environmental effects be further reduced before assuming that adverse ecological effects are unlikely to occur.

ACKNOWLEDGEMENTS

This study was supported by the E. I. duPont de Nemours and Company Educational Foundation; the Mobil Foundation; grant BSR-8705445 from the National Science Foundation; and by grant R-813805-01-0 from the Environmental Protection Agency.

We are grateful to Julie Yim for excellent technical assistance and to Darla Donald for preparing the manuscript for publication.

REFERENCES

[1] Strauss, H.S., Hattis, D., Page, G., Harrison, K., Vogel, S. and Caldarf, C. (1984) Genetically-engineered microorganisms: II. Survival, multiplication and genetic transfer. Recomb. DNA Tech. Bull. 9, 69-88

[2] Roszak, D.B. and Colwell, R.R. (1987) Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51, 365-379.

[3] Drahos, D.J., Barry, G.F., Hemming, B.C., Brandt, E.J., Skipper, H.D., Kline, E.L., Kluepfel, D.A., Hughes, T.A. and Gooden, D.T. (1988) Pre-release testing procedures: US field test of lacZY-engineered soil bacterium, in The Release of Genetically-Engineered Micro-Organisms (Sussman, M., Collins, C.H., Skinner, F.A. and Stewart-Tull, D.E., Eds.) pp. 181-191. Academic Press, New York.

[4] McCormick, D. (1988) Detection technology: the key to environmental microbiology. Bio/Technology 4, 419-422.

[5] Scanferlato, V.S., Orvos, D.R., Lacy, G.H. and Cairns, J., Jr. (in press) Enumerating low densities of genetically-engineered *Erwinia carotovora* in soil. Let. Appl. Microbiol.

[6] Cairns, J., Jr. and Pratt, J.R. (1986) Ecological censequence assessment: effects of bioengineered microorganisms. Water Res. Bull. 22, 171-182.

[7] Tiedje, J.M., Colwell, R.K., Grossman, Y.L., Hodson, R.E., Lenski, R.E., Mack, R.N. and Regal, P.J. (1989) The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. Ecology 70, 298-315.

[8] Alexander, M. (1971) Microbial Ecology. Wiley, New York.

[9] Stacey, G. (1985) The *Rhizobium* experience, in Engineered Organisms in the Environment: Scientific Issues (Halvorson, H.O., Pramer, D. and Rogul, M., Eds.), pp. 109-121. American Society for Microbiology, Washington, D.C.

[10] Wetzel, R. G. (1983) Limnology, 2nd ed. W.B. Saunders, Philadelphia.

[11] Cairns, J., Jr. and Pratt, J.R. (1986) Factors affecting the acceptance and rejection of genetically altered microorganisms by stablished natural aquatic communities, in Aquatic Toxicology and Environmental Fate: Ninth Symposium, ASTM STP 921 (Poston, T.M. and Purdy, R., Eds.) pp. 207-221. American Society for Testing and Materials, Philadelphia.

[12] Suter, G. W., II. (1985) Application of environmental risk analysis to engineered organisms, in Engineered Organisms in the Environment: Scientific Issues (Halvorson, H.O., Pramer, D. and Rogul, M., Eds.), pp. 211-219. American Society for Microbiology, Washington, D.C.

[13] Scanferlato, V.S., Orvos, D.R., Cairns, J., Jr. and Lacy, G.H. (1989) Genetically-engineered *Erwinia carotovora* in aquatic microcosms: Survival and effects on functional groups of indigenous bacteria. Appl. Environ. Microbiol. 55, 1477-1482

[14] Harrison, M.D., Franc, G.D., Maddox, D.A., Michaud, J.E. and McCarter-Zorner, N.J. (1987) Presence of *Erwinia carotovora* in surface water in North America. J. Appl. Bacteriol. 62, 565-570

[15] Maddox, D.A. and Harrison, M.D. (1988) Presence and population dynamics of *Erwinia carotovora* in irrigation water in south central Colorado. J. Appl. Bacteriol. 64, 169-182.

[16] Kotoujansky, A. (1987) Molecular genetics of pathogenesis by soft-rot erwinias. Ann. Rev. Phytopathol. 25, 405-430.

[17] Allen, C., Stromberg, V.K., Smith, F.D., Lacy, G.H. and Mount, M.S. (1986) Complementation of an *Erwinia carotovora* subsp. *carotovora* protease mutant with a protease-encoding cosmid. Mol. Gen. Genet. 202, 276-279.

[18] APHA, AWWA, WPCF (1985) Biological examination of water, in Standard Methods for the Examination of Water and Wastewater (Franson, M.A.H., Ed.) pp. 1041-1215. American Public Health Association, Washington, D.C.

[19] Owens, T.G. and King, F.D. (1975) The activity measurement of respiratory electron-transport-system activity in marine zooplankton. Marine Biol. 30, 27-36

[20] de Boer, H.S. (1982) Survival of phytopathogenic bacteria in soil, in Phytopathogenic Prokaryotes, vol.1. (Mount, M.S. and Lacy, G.H., Eds.) pp. 88-108. Academic Press Inc., New York.

[21] Liang L.N., Sinclair J.L., Mallory L.M. and Alexander, M. (1982) Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl. Environ. Microbiol. 44, 708-714

[22] Stromberg, V.K., Orvos, D.R., Scanferlato, V.S., Lacy, G.H. and Cairns, J., Jr. (in press) *In planta* competition among cell-degrading enzyme mutants and wildtype strains of *Erwinia carotovora* , in Proceedings of the 7th International Conference on Plant Pathogenic Bacteria. Hun. Acad. Sci.

[23] Lacy, G.H., Lamb, R.C. and Berg, C.M. (1981) Iris soft rot caused by *Erwinia chrysanthemi*, associated with overhead irrigation and its control by chlorination, in

Vol. 31 International Plant Propagators' Society Combined Proceedings for 1981, 31, 624-634.

[24] Jorge, P.E. and Harrison, M.D. (1986) The association of *Erwinia carotovora* with surface water in Northeastern Colorado. I. The presence and population of the bacterium in relation to location, season and water temperature. Am. Potato J. 63, 517-531.

[25] Brock, T. (1985) Procaryotic population ecology, in Engineered Organisms in the Environment: Scientific issues (Halvorson, H.O., Pramer, D., Rogul, M., Eds.) pp. 177-179. American Society for Microbiology. Washington, D.C.

[26] Martinez, J., Garcia-Lara, J. and Vives-Rego, J. (1989) Estimation of *Escherichia coli* Mortality in seawater by the decrease in ^{3}H-label and electron transport system activity. Microb. Ecol. 17, 219-225.

Chapter 5

Effect of sediment-associated copper on structure and function of aquatic microcosms *

SUMMARY

Fate and effect of sediment-associated copper (Cu) on the structure and function of aquatic communities were studied in aquatic microcosms. Sediment was spiked with nominal Cu concentrations of 0, 10, 100, or 1000 mg/kg dry sediment. Copper partitioning between sediment particles, pore water, and overlying water was followed in a 8-week long experiment. Most of the added Cu was bound to sediment particles. There was no evidence

* This chapter has been submitted to Aquatic Toxicology with the authors V. S. Scanferlato and J. Cairns, Jr.

that the structure or function of the community were affected in microcosms containing the nominal concentration of 10 mg Cu/kg sediment. In 100 mg/kg microcosms, with Cu concentrations of about 0.5 mg/l of overlying water, chlorophyll *a* content and respiration significantly decreased compared to the control. Failure to detect significant impact on other structural and functional attributes probably resulted from the high variability of the nontaxonomic attributes and the displacement of species sensitive to Cu by the tolerant species. In microcosms containing nominal concentration of 1000 mg Cu/kg (about 20 mg Cu/l of overlying water), the community showed a significant decrease in production, respiration, respiration/biomass ratio, ATP, and chlorophyll *a;* assimilation ratio and autotrophic index were significantly higher than in the control. Results of this study may be useful in predicting the impact of similar toxic stress on natural ecosystems.

INTRODUCTION

Sediments containing high levels of heavy metals represent a source of pollutants to aquatic organisms (Moore and Ramamoorthy, 1984; Chapman, 1989; Giblin, 1982). Contaminated sediments can disrupt structure and function of the aquatic communities, often resulting in losses of biological resources (Hodson et al., 1979; Malueg et al., 1984). Determining the impact of contaminated sediments is a key element in environmental risk assessment and management of water resources.

Sediments are often contaminated with copper (Cu) that originates from discharges of mine tailings, fly ash, and wastes of the metal industry (Helz, 1976; Moore and Ramamoorthy, 1984). Deleterious effects of Cu on aquatic communities are well documented (Winner et al., 1972; Hodson et al., 1979; Hedtke, 1984). Because of its toxic properties, Cu has been widely used as algicide and molluscicide (Fitzgerald, 1975; Hodson, 1979).

When discharged into the water column, heavy metals enter into complexation and sorption reactions with the solid phase (Jennet and Effler, 1980). Sediment has thus been characterized as a "sink" to trace metals in freshwater ecosystems. Changes in the chemical environment can alter the solid-solution equilibria and cause the re-entry of heavy metals into the dissolved state (Duinker and Nolting, 1977). Copper from sediments can therefore affect organisms that are not in direct contact with the contaminated substrate. Bioavailability of Cu adsorbed to sediments, factors that control its toxicity, and the extent to which Cu solubilizes from sediments and affects aquatic life in overlying water are not well understood.

Microcosms were used in this study as surrogates of natural ecosystems in order to evaluate the toxicity of sediment-associated Cu to aquatic communities. Microcosms integrate the responses of the higher levels of biological organization to toxic stress, have the advantage of replicability and eliminate the environmental damage that might occur if natural systems were exposed. They allow measurement of ecosystem-level properties that arise from a set of interactions within the system, which cannot be done at lower levels of biological organisation (Kimball and Levin, 1985). The microcosm approach also takes into account the reactions of Cu with abiotic and biotic components of the system that may mediate concentration and chemical speciation and, thus, the toxicity of Cu to aquatic organisms (Morel et al., 1988; Verweij et al., 1989).

The objective of this study was to assess risks associated with contaminated sediments by 1) following Cu partitioning into various compartments of the system and 2) assessing the impact of sediment-associated Cu on community structure (ash-free dry weight, chlorophyll *a*, autotrophic index, ATP content) and function (production, assimilation ratio, respiration, production/respiration, production/biomass, respiration/biomass). The results may be useful in predicting the responses of natural aquatic ecosystems to a similar toxic stress.

MATERIALS AND METHODS

Microcosms

Microcosms were constructed in 25-l glass aquaria that were divided into two parts by a plexiglass partition. Sediment was placed into one part of the aquarium, and water was added into the other until it overflowed the partition and covered the sediment (Fig. 1). Sediment and water were collected at Pandapas Pond, Montgomery County, Virginia. Characteristics of dilution water were: pH, 6.8; hardness, 15 mg/l $CaCO_3$; alkalinity, 11 mg/l $CaCO_3$; conductivity, 94 μmhos. Sediment characteristics were: pH, 5.7; organic matter, 0.8%; total phosphorus, 2 mg/kg; nitrate, 3 mg/kg; particle sizes: sand, 47.4%; silt, 30.4%; clay, 22.2%.

A total of 16 microcosms was established to test effects of four Cu concentrations in four replicate microcosms. The sediment was collected, drained, and sieved through a 5-mm screen. Each microcosm received 15.15 kg of wet sediment (equivalent to 12 kg dry sediment, or 6.9 l). Copper was applied to sediment within 24 h in the form of $CuSO_4$ $5H_2O$ dissolved in 250 ml of distilled water. Copper concentrations were adjusted to deliver nominal concentrations of 0, 10, 100, and 1000 mg Cu/kg dry sediment. The spiked sediment was thoroughly mixed and allowed to equilibrate with copper sulfate for 24 h before pouring 16 l of water into each of the microcosms. Microcosms were kept at 21 $\pm$ 2°C on a 16-h light cycle. Light intensity on the water surface was 30 μEinsteins/m^2/s. The experiment lasted 56 days and the samples were taken weekly. To replace water lost by evaporation and provide an inoculum source, water from Pandapas Pond was added to microcosms weekly. Sediment samples were obtained by inserting a 10-ml plastic pipette with its conical tip removed through the depth of the sediment (a 0.6 cm^2 core). Samples of overlying water were taken with a plastic syringe

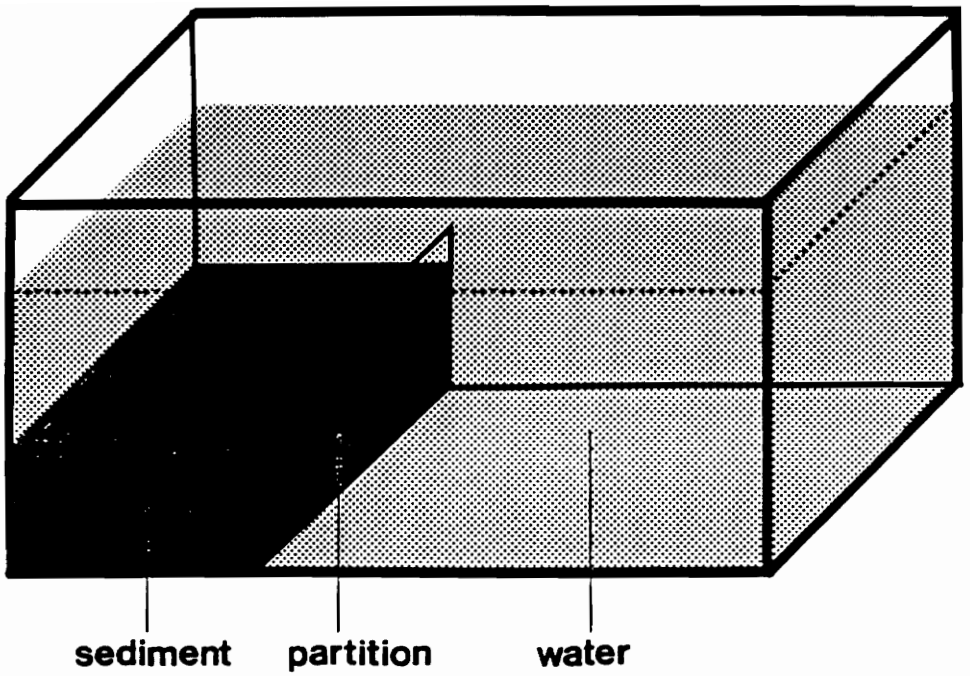

Fig. 1. Schematic diagram of sediment-water microcosms used in the experiment.

after scraping the algae from the sides of the container, since the heavy algal side growth accounted for much of the activity in the microcosms.

Copper analysis

Samples for Cu analyses of bulk sediment, pore water, and overlying water were taken 30 minutes after addition of water over the spiked sediment and weekly afterwards. Concentrations of Cu in bulk sediment were determined by digesting sediment samples (1 g dry weight) in 15 ml of 1:1 nitric and sulphuric acid. The solution was boiled to near dryness over a period of 4 h, cooled, diluted with distilled deionized water up to 50 ml, and filtered through a 0.45-μm membrane filter. Concentration of dissolved Cu in pore water was determined by centrifuging a 15-ml sediment sample at 3000 x *g* for 1 h. The supernatant was filtered through 0.45-μm membrane filters, transferred into a plastic tube, acidified with nitric acid, and stored at 4°C. To determine Cu concentrations in overlying water, samples were passed through a 0.45-μm membrane filter, acidified, and stored in plastic tubes at 4°C. Copper concentrations ranging between 0.2 and 4 mg Cu/l were analyzed by flame atomic absorption spectrophotometry on Perkin-Elmer Model 1100. Copper concentrations ranging between 5 and 50 μg Cu/l were analyzed on a Perkin-Elmer HGA-300 graphite furnace atomic absorption spectrophotometer.

Functional parameters

Production and respiration were estimated from the rates of oxygen evolution and uptake in dark and light bottles (APHA et al., 1985). To determine production and respiration of the community that developed in overlying water, bottles were filled with water-periphyton suspension. After measuring the initial oxygen concentrations, bottles were sealed and incubated for 8 h in light or dark, and the oxygen measurements were repeated.

Assimilation ratio (AR) indicates the photosynthetic oxygen production per unit plant material, i.e. the relative productivity of photosynthetic assemblage. It is calculated as:

$$AR = \text{net hourly production (mg } O_2\text{/h/l)/ chlorophyll } a \text{ (mg/l)}$$

The production/respiration (P/R) ratio has been suggested as an indicator of ecosystem stress. According to Odum (1985), an increase in respiration greater than the increase in production usually follows the disturbance.

Community production and respiration per unit biomass (P/B and R/B, respectively) have also been suggested as indicators of stress. It is hypothesized that R/B ratio should increase when the system is exposed to stress, because the community is "pumping out" disorder (Odum, 1985); P/R ratio is expected to become unbalanced with stress.

Structural parameters

Ash-free dry weight (AFDW) represents an estimate of total community biomass (autotrophic, heterotrophic, and detrital). Water samples for AFDW measurements were collected with a 50-ml plastic syringe. The contents of the syringe were filtered onto preashed and preweighed glass-fiber filter (type GFF/A, effective pore size= 1.6 μm). Filters were dried at 105°C for 24 h, cooled for 2 h in the dessicator, and weighed. Filters were then ashed in a muffle furnace at 550°C for 1 h, cooled, sprinkled (wetted) with distilled water, dried at 105°C for another 24 h, and weighed again. The difference between the two measurements is AFDW (APHA et al., 1985).

Chlorophyll *a* concentration, an estimate of biomass of autotrophic organisms, was measured by standard methods (APHA et al., 1985). Water samples of 50 or 100 ml were placed into tubes and centrifuged at 1800 x g for 20 minutes. The pellet was re-suspended in 5 ml of 90% buffered aqueous acetone and extracted overnight at 4°C in the dark. Samples were centrifuged and the absorbance was measured at 750 nm and

664 nm. To correct for phaeopigments, 0.1 ml of 0.1 M HCl was added to the cuvette. The absorbance was measured after 90 sec at 750 nm and 665 nm.

Autotrophic index (AI) indicates the trophic nature of the community. It is calculated as:

$$AI = \text{AFDW (mg/l)} / \text{chlorophyll } a \text{ (mg/l)}$$

Measurement of ATP content provides an estimate of total living biomass. ATP was assayed with the luciferin-luciferase bioluminiscence technique as described in Karl and LaRock (1975). Water samples (20, 50 or 100 ml) were collected with a plastic syringe and filtered onto GFF/A. Filters were placed into glass tubes. To extract ATP, 5 ml of 0.6 N H_2SO_4 were added and the filters were macerated with a glass rod. Tris buffer was added after 10 minutes and the contents were vortexed. Samples were centrifuged for 5 minutes at 1800 x *g*, and 4 ml of supernatant from the each tube were transferred into a beaker. One ml of 0.048 M EDTA made up in Tris was added; pH was adjusted to 7.8 with NaOH; and the volume was adjusted with Tris to r10 ml. Contents were transferred into plastic graduated tubes, stoppered, and immediatelly frozen. ATP levels in samples were quantitatively determined using Fireflight, the firefly luciferin-luciferase enzyme assay (Analytical Luminescence Laboratory Inc., San Diego, CA 92121). All assays were made with a Labline 9140 ATP Photometer. The ATP standard curve and the difference between background and sample lumination were used to calculate each ATP concentration.

Experimental design and statistical analysis

Repeated measures experimental design was used. Four blocks were established, each consisting of four microcosms containing four different concentrations of sediment-associated Cu. Blocking factor was location of microcosms. One-tailed Dunnett testing procedure was used to determine whether the mean of the control group differed signif-

icantly from each of the means of the other groups. Statements relative to statistical significance are at the 0.05 probability level or less.

RESULTS

Copper partitioning

Copper concentrations in bulk sediment are shown on Table I. Copper concentrations in bulk sediment were significantly higher in microcosms receiving nominal concentrations of 100 and 1000 mg Cu/kg sediment than in control microcosms; however, the differences on individual days between the control and 100 mg Cu/kg microcosms were statistically significant only on day 49.

Pore water concentration increased with increasing concentrations of Cu added to sediment (Table II). The interaction between Cu concentration and time was significant. On individual days, significant differences between pore water concentrations in microcosms spiked with 100 mg/l and in control microcosms could not be detected; the exception was day 56. In microcosms in which sediment was spiked with the nominal concentration of 1000 mg/kg, pore water concentrations were significantly higher.

Copper concentrations in overlying water are presented on Table III. There was a significant interaction between Cu concentration in overlying water and time. In microcosms exposed to 10 and 100 mg Cu/kg sediment, Cu concentrations in the water column on individual days were not significantly different from the control. In microcosms exposed to 1000 mg/l, concentrations in the water column were significantly higher than in the controls.

TABLE I
Mean copper (Cu) concentrations in bulk sediment

Nominal concentration of sediment-associated Cu (mg/kg)	Cu concentration (ug/l)								
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
0	6.58 (1.91)[a]	4.96 (1.58)	2.75 (0.25)	2.37 (0.49)	7.85 (2.28)	6.43 (1.89)	5.65 (0.95)	6.88 (1.43)	9.14 (1.20)
10	18.31 (12.01)	11.97 (1.69)	8.32 (0.31)	8.84 (4.50)	12.12 (4.37)	22.58 (7.11)	18.67 (6.56)	15.53 (2.86)	15.58 (1.47)
100	69.02 (27.77)	80.77 (16.09)	70.41 (16.01)	88.54 (24.04)	81.86 (33.91)	67.07 (19.89)	72.42 (14.48)	73.79★ (9.72)	83.67 (18.26)
1000	737.28★[b] (189.31)	722.83★ (171.64)	726.20★ (142.25)	810.21★ (281.90)	767.45★ (141.07)	846.46★ (197.28)	747.09★ (166.02)	697.15★ (32.88)	738.55★ (106.28)

[a]Values in parentheses are standard deviations; four replicates for day 0 and 7; three replicates for days 14 to 56.
[b]Means with an asterisk are significantly different from the control (Dunnett's testing procedure)

TABLE II
Mean copper (Cu) concentrations in pore water

Nominal concentration of sediment-associated Cu (mg/kg)	Cu concentration (ug/l)								
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
0	12 (5)[a]	17 (3)	15 (4)	17 (6)	12 (3)	14 (2)	10 (4)	12 (2)	16 (2)
10	12 (6)	19 (7)	16 (2)	26 (4)	20 (3)	19 (7)	23 (12)	21 (5)	24 (9)
100	674 (322)	456 (267)	1140 (684)	890 (508)	1219 (617)	637 (305)	724 (181)	599 (173)	466★ (172)
1000	423883★[b] (108494)	142089★ (63469)	167177★ (51761)	177036★ (31734)	221669★ (87214)	170600★ (47235)	165000★ (33941)	205350★ (54659)	- (-)

[a]Values in parentheses are standard deviations; four replicates for day 0 and 7; three replicates for days 14 to 56
[b]Means with an asterisk are significantly different from the control (Dunnett's testing procedure)

TABLE III
Mean copper (Cu) concentrations in water

Nominal concentration of sediment-associated Cu (mg/kg)	Cu concentration								
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
0	< 5 (-)	< 5 (-)	6 (1)	< 5 (-)	< 5 (-)	< 5 (-)	< 5 (-)	< 5 (-)	< 5 (-)
10	< 5 (-)	6 (1)	7 (1)	8 (1)	9 (1)	6 (-)	< 5 (-)	< 5 (-)	< 5 (-)
100	21 (8)[a]	643 (45)	817 (212)	508 (185)	783 (247)	430 (137)	397 (144)	363 (95)	327 (55)
1000	4280★[b] (1963)	9825★ (3513)	8250★ (2709)	19100★ (7637)	29800★ (8374)	21910★ (9603)	19650★ (11102)	28050★ (16758)	32300★ (20365)

[a]Values in parentheses are standard deviations; four replicates for day 0 and 7; three replicates for days 14 to 56.
[b]Means with an asterisk are significantly different from the control (Dunnett's testing procedure)

Effect on community function

The effect of Cu on production of aquatic communities that developed in overlying water is shown on Fig. 2A. Interaction between concentration and time was significant. Production was significantly lower in microcosms with nominal concentrations of sediment-associated Cu of 1000 mg/kg from day 14 to day 28. From day 35 to day 56, differences were not significant, although the means in the 1000 mg/kg microcosms appeared to be lower than in the control.

Figure 2B shows the effect of Cu on the assimilation ratio in overlying water. Interaction between concentration and time was significant. Communities from microcosms containing nominal concentrations of 1000 mg Cu/kg sediment had significantly higher assimilation ratios, i.e., produced more oxygen per unit algal biomass than those in control microcosms at days 14, 21, 42, and 49. On the other days, the mean assimilation ratios were higher than in the control microcosms, but the differences between them were not significant.

Respiration in overlying water is shown on Fig. 2C. There was a significant interaction between Cu concentration and time. In microcosms containing nominal concentrations of 1000 mg Cu/kg sediment, respiration was significantly lower than in the control during the whole experiment, with the exception of day 14. Exposure to Cu in the 100 mg/kg microcosms resulted in significantly lower respiration values than in the control between days 35 and 56. Respiration increased until day 35 in microcosms exposed to 0, 10, and 100 mg Cu/kg, but remained depressed and relatively stable in microcosms containing the nominal concentration of 1000 mg Cu/kg.

Production/respiration ratio (Fig. 2D) was not significantly different among microcosms exposed to different Cu treatments; the effect of time was significant. There was a similar trend in microcosms containing 0, 10, and 100 mg Cu/kg sediment: P/R

steadily increased, reached its peak at day 14, and then declined. In microcosms treated with 1000 mg Cu/kg, P/R increased from day 21 to day 35 and then declined.

The production/biomass ratio is shown on Fig. 2E. The interaction between Cu concentration and time was significant. The P/B was significantly depressed at days 14 and 21.

The effect of Cu concentration and time on the R/B ratio (Fig. 2F) was significant. In microcosms exposed to nominal concentrations of 1000 mg Cu/kg sediment, there was a significant decrease in R/B ratio.

Effect on community structure

Effect of time on AFDW of the community in overlying water was significant (Fig. 3A). The AFDW increased over time, regardless of the Cu concentration to which the community was exposed. There was a significant increase of AFDW content in 1000 mg/kg microcosms between days 7 to 21. Mean AFDW appeared to be lower in 1000 mg/kg microcosms than in the control during the second part of the experiment, but the differences were not statistically significant.

The effects of Cu concentration and time on chlorophyll *a* concentration in overlying water were significant. Both in 100 mg/kg and 1000 mg/kg microcosms, chlorophyll a concentration was significantly lower than in the control. Chlorophyll concentration sharply increased in microcosms with nominal concentrations of 0, 10, and 100 mg Cu/kg; in 1000 mg Cu/kg microcosms, chlorophyll concentrations remained depressed (Fig. 3B).

The values of autotrophic indices are shown on Fig. 3C. Interaction between Cu concentration and time was significant. On individual days, AI was significantly higher in microcosms exposed to 1000 mg/kg than in control microcosms throughout the experiment, except on day 42.

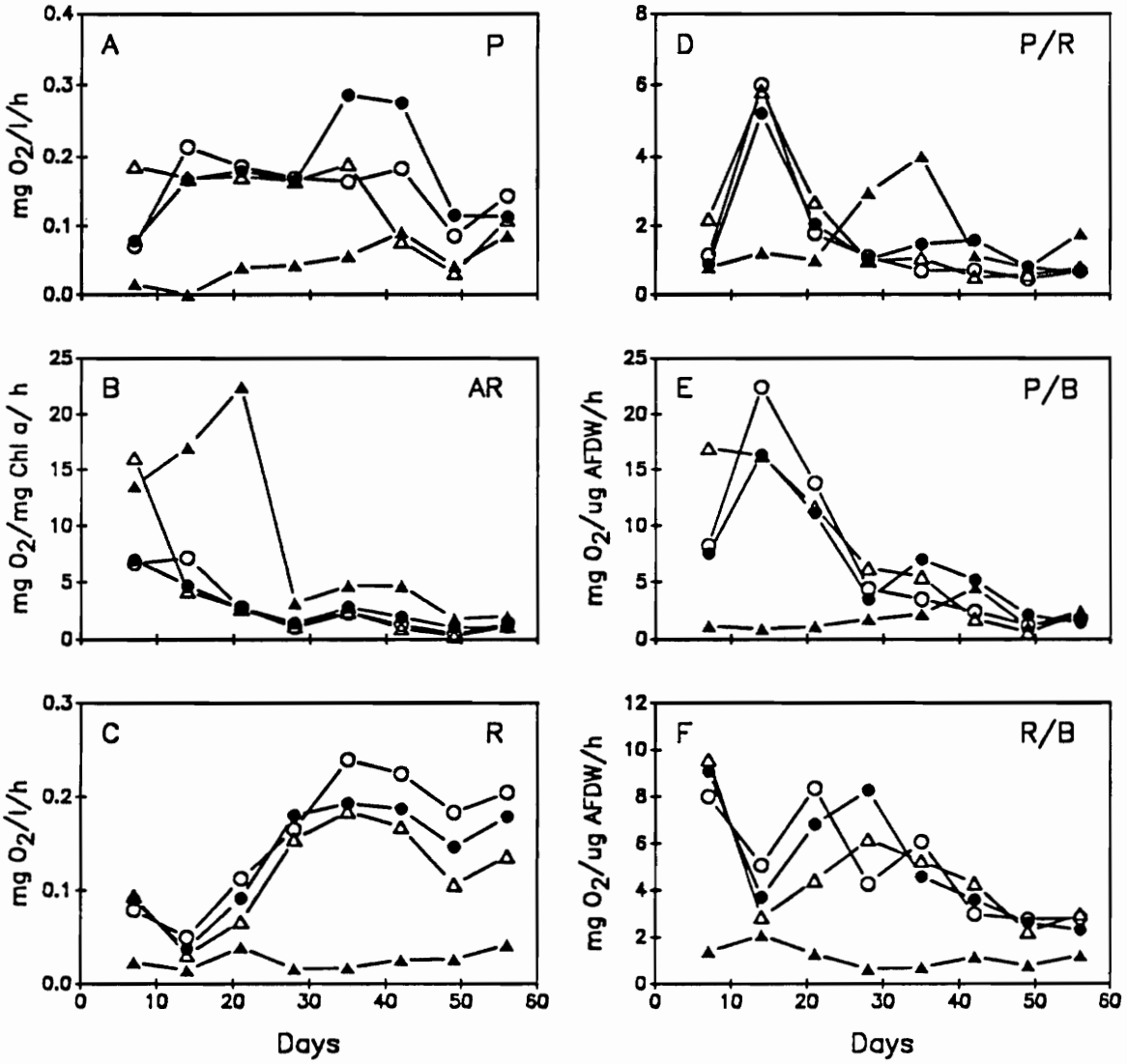

Fig. 2. Effect of sediment-associated copper on microcosm functional properties: production (A), assimilation ratio (B), respiration (C), production/respiration ratio (D), production/biomass (E), and respiration/biomass ratio (F). Sediment was spiked with copper concentrations of 0 mg/kg (○), 10 mg/kg (•), 100 mg/kg (△), and 1000 mg/kg (▲).

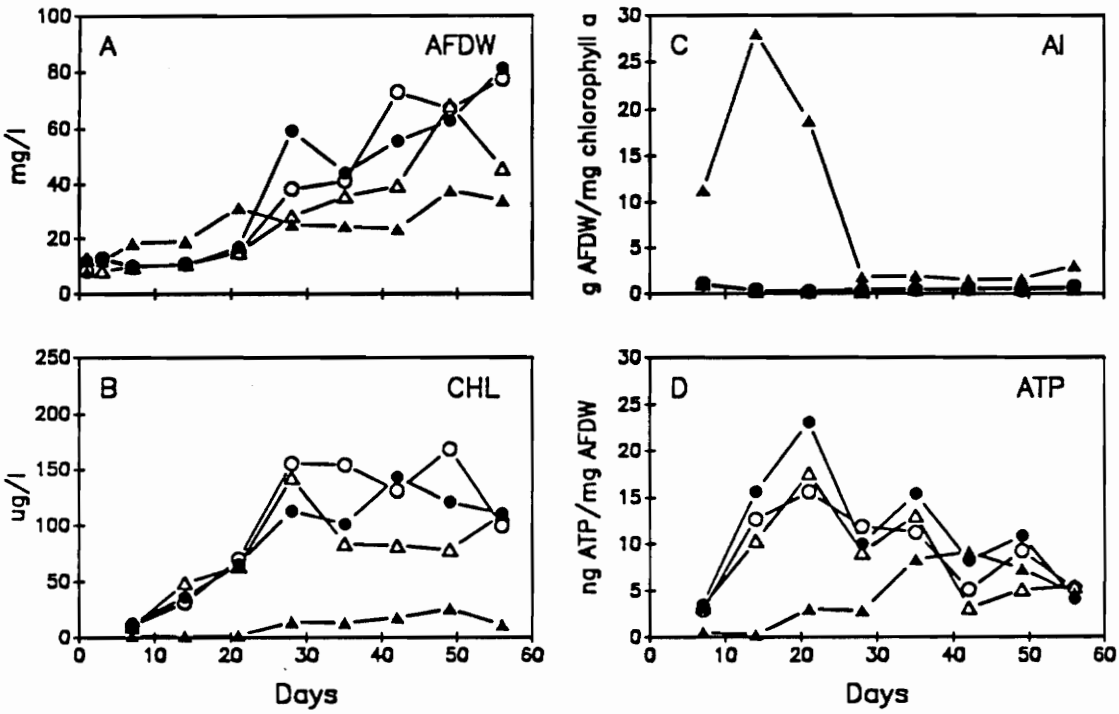

Fig. 3. Effect of sediment-associated copper on microcosm structural properties: ash-free dry weight (A), chlorophyll a (B), autotrophic index (C), and ATP concentrations (D). Sediment was spiked with copper concentrations of 0 mg/kg (○), 10 mg/kg (•), 100 mg/kg (△), and 1000 mg/kg (▲).

Copper concentration and time had a significant effect on ATP content in overlying water (Fig. 3D). The ATP concentrations were significantly lower in microcosms with nominal concentrations of 1000 mg Cu/kg sediment than in the control microcosms.

DISCUSSION

Copper analysis showed a partitioning of the added Cu into the solid phase where it was bound to sediment particles. Pore water accounted for 20% of the sediment weight, but contained less than 1% of the total sediment-associated Cu in microcosms spiked with 10 and 100 mg Cu/kg. In microcosms spiked with 1000 mg Cu/kg sediment, pore water accounted for 20% of the total copper. The Cu concentration in pore water increased with increasing copper concentration added to the sediment. Since heavy metals are often found to be preferentially associated with the smaller particle sizes and organic particles (Jennett and Effler, 1980), it seems likely that the high clay and silt content of sediment (22.2% and 30.4%, respectively) promoted partitioning of Cu into the solid phase. In sediments with a clay and silt content of 11.8% and 47.5%, respectively, over 99% of the added Cu was reported to be chemically and physically bound to sediment particles (Cairns et al., 1984).

Nontaxonomic structural and functional measures provided an insight into the response of the aquatic community to Cu. Perturbations were observed in microcosms exposed to nominal concentrations of 1000 mg Cu/kg sediment, which contained about 20 mg Cu/l in overlying water. Production, respiration, and R/B ratio were lower in these microcosms than in the control, while the AR and AI significantly increased. The AFDW was significantly higher in the first part of the experiment than in the control. The reasons for this are not clear, since the increase was not due to the living biomass; in the same microcosms, ATP levels were significantly reduced. A possible explanation

for the observed discrepancy is the response of bacteria to stress by excretion of mucilage; they probably contributed to an increase of community AFDW, without a simultaneous increase of ATP content. Depressed production can be related to the low chlorophyll *a* content. A decrease in respiration probably resulted from elimination of organisms due to the high concentrations of Cu. Reduced respiration was also observed by Hedtke (1984) after the exposure of the aquatic community to Cu. Decreased or unchanged respiration and R/B ratio in our microcosms exposed to Cu do not support the hypothesis of Odum (1985) that increased respiration indicates stress. Zinc stress was also observed to decrease rather than increase the R/B ratio (B. Niederlehner, personal communication). The AI was higher in microcosms exposed to 1000 mg/kg sediment than in the control, showing that the system became primarily heterotrophic. The high AI values resulted from the nonviable organic material, which affects this index by inflating the numerator.

In microcosms with the nominal concentration of sediment-associated Cu of 100 mg/kg (about 0.5 mg/l of overlying water), chlorophyll *a* concentration and respiration were the only parameters that significantly differed from the control. The absence of the measurable effect on the majority of structural and functional measures may be due to the presence of factors that mediate the toxicity of Cu in water. Bioavailability of copper is governed by its chemical speciation; free Cu (Cu_{aq}^{2+}) controls the bioavailability and toxicity of Cu. Adsorption/desorption, precipitation/dissolution, and association/dissociation of inorganic and organic complexes determine the properties of the free Cu (Verweij et al., 1989). The presence of natural organic compounds that have complexing properties can reduce the toxicity of Cu (Cairns et al., 1984; Hodson et al., 1979; Morel et al., 1988; Verweij et al., 1989). Since the toxicity of Cu is primarily attributed to free ions, organic compounds present in our microcosms may have complexed the Cu, altering its toxicity to aquatic organisms.

High variability of nontaxonomic structural and functional parameters precluded the detection of statistically significant differences in 100 mg/kg microcosms, even when the mean values appeared different. This suggests that a higher number of replicates should be used in order to detect significant differences. Rogers et al. (1980) found functional values measured during their experiments statistically less variable than structural values. However, the nontaxonomic structural parameters used in their study are also considered to be highly variable. Most authors believe that the inherent variability of community functional measures may limit their use in detecting ecosystem damage (Crossey and La Point, 1988; Schindler, 1987).

The lack of a statistically significant impact in 100 mg/kg microcosms on the majority of the structural and functional parameters may be due to the displacement of species sensitive to Cu by the tolerant species. The loss of species may have occurred without a significant alteration of community function (Cairns and Pratt, 1986). Phytoplankton assemblages can perform primary ecosystem function over a wide range of conditions, even after the change in species composition occurs (Schindler, 1987). Since functional redundancy occurs in the major attributes of an aquatic community, species tolerating high Cu concentrations, such as *Synura, Dynobryon,* and *Achnanthes* (Whitton, 1971), may have started performing the functions of those organisms that were displaced by the toxic stress. If changes in taxonomic structure occurred in our study, they would not be detected because the measurements of community structure and function were nontaxonomic.

Results of this study may be useful in predicting the effects of sediments contaminated with Cu on natural aquatic ecosystems and designing the water quality standards to protect the structure and function of the aquatic communities. Our data show that concentrations of sediment-associated Cu of 1000 mg/kg affected nearly all aspects of structure and function in aquatic microcosms. However, concentrations of 100 mg

Cu/kg sediment did not have a significant effect on the majority of the measured parametes. The nontaxonomic measurements seem to be too insensitive to be considered effective tools for environmental impact evaluation. Data obtained in our research could probably lead to standards protecting the nontaxonomic attributes of community structure and function, but not necessarily to protecting the taxonomic structure of the aquatic communities in the receiving systems.

ACKNOWLEDGMENTS

This study was supported by E. I. du Pont de Nemours & Company Educational Foundation and the Mobil Foundation. We are grateful to B. Niederlehner for helpful suggestions and comments on the draft, to E. Smith for his help in statistical analysis, to J. Yim for technical assistance, and to Darla Donald for preparing the manuscript for publication.

REFERENCES

APHA, AWWA, WPCF, 1985. Biological examination of water. In: Standard methods for the examination of water and wastewater, edited by M.A.H. Franson, American Public Health Association, Washington, DC, pp. 1041-1215

Cairns, J., Jr. and J.R. Pratt, 1986. On the relation between structural and functional analyses of ecosystems. Environ. Toxicol. Chem. 5, 785-786.

Cairns, M.A., A.V. Nebeker, J.H. Gakstatter and W.L. Griffis, 1984. Toxicity of copper-spiked sediments to freshwater invertebrates. Environ. Toxicol. Chem. 3, 435-445.

Chapman, P.M., 1989. Current approaches to developing sediment quality criteria. Environ. Toxicol. Chem. 8, 589-599.

Crossey, M.J. and T.W. La Point, 1988. A comparison of periphyton community structural and functional responses to heavy metals. Hydrobiologia 162, 109-121.

Duinker, J.C. and R.F. Nolting, 1977. Dissolved and particulate trace metals in the Rhine Estuary and the Southern Bight. Mar. Pollut. Bull. 8, 65-71.

Fitzgerald, G.P., 1975. Are chemicals used in algae control biodegradable? Water Sewage Works 82-85.

Giblin, A.E., 1982. Uptake and remobilization of heavy metals in salt marches, Ph.D. Dissertation, Boston University Graduate School, University Microfilms International, Ann Arbor, MI, 283 pp.

Hedtke, S.F., 1984. Structure and function of copper-stressed aquatic microcosms. Aq. Toxicol. 5, 227-244.

Helz, G.R., 1976. Trace element inventory for the northern Chesapeake Bay with emphasis on the influence of man. Geochim. Cosmochim. Acta 40, 573-580.

Hodson, P.V., U. Borgmann and H. Shear, 1979. Toxicity of copper to aquatic biota. In: Copper in the environment, edited by J. O. Nriagu, John Wiley & Sons, New York, pp. 307-372.

Jennett, J.C. and S.W. Effler, 1980. Mobilization and toxicological aspects of sedimentary contaminants. In: Contaminants and sediments vol. 1, edited by R. A. Baker, Ann Arbor Science, Ann Arbor, MI, pp. 429-444.

Karl, D.M. and P.A. LaRock, 1975. Adenosine triphosphate measurements in soil and marine sediments. J. Fish. Res. Board Can. 32, 599-607.

Kimball, K.D. and S.A. Levin. 1985. Limitations of laboratory bioassays: the need for ecosystem-level testing. BioScience 35, 165-171.

Malueg, K.W., G.S. Schuytema, J.H. Gakstatter and D.F. Krawczyk, 1984. Toxicity of sediments from three metal-contaminated areas. Environ. Toxicol. Chem. 3, 279-291.

Moore, J.W. and S. Ramamoorthy, 1984. Copper. In: Heavy metals in natural waters. Springer-Verlag, New York, pp. 77-99.

Morel, J.L., G. Bitton and B. Koopman, 1988. Use of Microtox for assessing copper complexation with organic compounds. Arch. Environ. Contam. Toxicol. 17, 493-496.

Odum, E.P., 1985. Trends expected in stressed ecosystems. BioScience 35, 419-422.

Rogers, J.H., Jr., K.L. Dikson and J. Cairns, Jr., 1980. A review and analysis of some methods used to measure functional aspects of periphyton. In: Methods and measurements of periphyton communities: a review, edited by R. L. Weitzel, American Society for Testing and materials, Philadelphia, PA, pp. 142-167.

Schindler, D. W., 1987. Detecting ecosystem responses to anthropogenic stress. Can. J. Fish. Aquat. Sci. 44, 6-25.

Verweij, W., H. De Haan, T. De Boer and J. Voerman, 1989. Copper complexation in eutrophic and humic Lake Tjeukemeer, The Netherlands. Freshwater Biol. 21, 427-436.

Whitton, B.A., 1971. Toxicity of heavy metals to freshwater algae: A review. Phykos 9, 116-125.

Winner, R.W., J. Scott Van Dyke, N. Caris and M.P. Farrel, 1972. Response of the macroinvertebrate fauna to a copper gradient in an experimentally-polluted stream. Appl. Limnol. 2121-2127

Chapter 6

Comparative evaluation of copper toxicity to *Simocephalus exspinosus* in single-species and microcosm tests

SUMMARY

This study compared the responses of individuals to copper (Cu) during a single-species test to the responses of populations of the same species in an ecologically more complex system. A routine single-species test was performed by exposing *Simocephalus exspinosus* (Daphnidae), to Cu concentrations of 0, 3, 10, 30, 100, and 300 μg/l. Results of this test were validated in aquatic microcosms. In both test systems, an increased production of neonates occurred after exposure of animals to 30 to 46 μg Cu/l. At these copper concentrations, instantaneous rates of population growth were higher than in the controls. The organisms did not survive at concentrations > 100 μg Cu/l during the single-species or

microcosm tests. The responses of *Simocephalus* exposed to copper appeared to be similar in both test systems.

INTRODUCTION

Laboratory tests involving single species have traditionally been used to gather information on the toxicity of specific substances and to predict responses of ecosystems receiving toxic materials. Toxicant concentrations that demonstrated no observable effect on a test species were considered safe. This afforded use of single-species toxicity tests to set water quality criteria for environmental management and protection purposes.

One of the major criticisms of laboratory tests is that they lack environmental realism, i.e., the differences between the natural system and the test system are great (Kimball and Levin, 1985). Test systems do not fluctuate in physical and chemical factors such as temperature, hardness, pH, and dissolved oxygen concentration; also, the complexation of a toxic substance or its uptake by algae in standard test systems may not correspond to similar processes which occur in natural systems (Hodson et al., 1979), all of which can mediate toxicity. Laboratory tests use genetically homogeneous laboratory-stock test populations that may lack the adaptive ability of natural populations (Pontash et al., 1989). In natural systems, populations are exposed to parasitism and predation; moreover, competition with species that are more resistant to toxicants may increase the susceptibility to toxic stress.

It has been shown that the laboratory bioassays can underestimate effects of a toxicant. Subsequent release of a toxic substance can have an adverse effect on a natural system (Roberts et al., 1978). Laboratory test results can also indicate much more subtle effects than those that occur in natural ecosystem; in that case, standards may

"overprotect" the system. For this reasons and others, extrapolation from responses of isolated organisms in single-species laboratory tests to responses of that same species in a natural ecosystem may not be accurate and justified (Cairns 1988 a, b; Kimball and Levin, 1985).

This study evaluated the predictive ability of standard tests on a single level of biological organization -- the population. It assessed how accurate it is to extrapolate from the responses of individuals to a toxic substance during a single-species test to the responses of populations of the same species in an ecologically more complex system.

A routine single-species test was performed to evaluate toxicity of copper (Cu) to *Simocephalus exspinosus* Koch (Daphnidae), a littoral cladoceran isolated from Airport Pond, Montgomery County, Va. This test was conducted using a *Simocephalus* strain maintained in the laboratory.

Results of this test were validated in aquatic microcosms, using natural *Simocephalus* populations from the same pond. Microcosms were used because they integrate the responses of higher levels of biological organization to toxic stress, provide intermediate complexity, have the advantage of replicability, and preclude environmental damage that might occur in exposing natural systems.

MATERIALS AND METHODS

Copper analysis

To determine concentrations of dissolved copper to which organisms were exposed during the test, test solutions were sampled weekly at the beginning and at the end of the renewal period. Samples were filtered through a 0.45 μm membrane filter, acidified, and analyzed by flameless techniques on a Perkin-Elmer HGA-300 graphite furnace atomic absorption spectrophotometer.

Single-species test

Laboratory stock culture used in single-species tests originated from natural *Simocephalus exspinosus* populations collected from the Airport Pond, Montgomery County, Va. The organism was cultured under controlled conditions for three months. *Simocephalus* neonates (< 24 h old) were exposed to a control and five concentrations of copper in the form of $CuSO_4$ $5H_2O$ (3, 10, 30, 100, and 300 μg Cu/l) prepared with Airport Pond water filtered through a 0.45 μm membrane filter. Ten beakers of each copper concentration received 30 ml of the test solution, and one test organism was placed into each beaker. The experiment lasted three weeks. Animals were fed daily with tri-algal diet (Belanger et al., 1989). Test solutions were renewed every 2 to 3 days, and the number of live and dead animals were recorded at those times. Neonates were discarded, and the surviving parent animal was transfered to a beaker containing the same copper concentration as in the one from which it was removed. The test was conducted at 25°C using a 16-hr light cycle.

Instantaneous rate of population growth (r) was calculated in single-species test for each exposure concentration as:

$$1 = L_x M_x e^{-rx}$$

where x is the age in days, L is the probability of survival to age x, and M is the number of female young per female produced during the surrounding time interval.

Microcosm test design

Predictions of copper toxicity to *Simocephalus* obtained in single-species tests was validated in microcosms. Microcosms were constructed in 850-ml glass jars filled with water collected from Airport Pond. Microcosms were incubated at 25°C and illuminated on a 16-hr light cycle (Horning and Weber, 1985). Four replicate microcosms were used for each copper concentration. One half the test solution was replaced twice

weekly by passing the upper 350 ml of the water column through a filter retaining particles > 35 μm. The filtrate was saved for analyses. New replacement solutions was prepared with Airport Pond water filtered through a 35 μm filter, and 350 ml of the appropriate solution was placed into each microcosm.

Instantaneous growth rates of natural populations in microcosm tests were calculated from the equation:

$$r = (\ln N_t - \ln N_i)/ \Delta t$$

where N_t is the average terminal number of *Simocephalus* in samples at a given Cu level for a given exposure of time, N_i is the average initial number, and Δt is exposure time in days.

Analysis of variance and least significant difference (LSD) test were used to evaluate statistical significance between microcosms exposed to different Cu treatments. Statements relative to the statistical significance are at the 0.05 probability level or less.

RESULTS AND DISCUSSION

Copper concentrations in single-species test and microcosm test are shown on Tables I and II, respectively. In single-species test, beakers with nominal concentrations of 0, 3, and 10 μg Cu/l contained higher Cu concentrations at the end, than in the beginning of the renewal period. Algae added as a food source for *Simocephalus* were probably responsible for contamination of water containing low Cu concentrations. Cu concentrations decreased at the end of the renewal period in beakers containing nominal concentrations of 100 and 300 μg Cu/l. Bioaccumulation of Cu by algae (Trollope and Evans, 1976) may explain a decrease of its concentration in beakers with the highest two Cu concentrations. In microcosm tests, Cu concentrations were always lower at the end, than in the beginning of the renewal period, probably due to the uptake of Cu by algae

TABLE I
Copper concentrations in single-species test

Nominal Cu concentration (ug/l)	Concentration of added Cu[a] (ug/l)	Cu concentrations at the end of renewal period (ug/l)[b] Week 1	Week 2	Week 3
0	< 5.00	6.64 (0.42)	9.49 (0.78)	12.63 (0.75)
3	< 5.00	7.84 (0.41)	12.46 (1.24)	14.13 (1.46)
10	10.53 (0.23)[c]	12.18 (1.35)	14.48 (1.42)	19.00 (1.04)
30	30.87 (0.58)	27.86 (3.10)	28.75 (0.94)	33.12 (2.07)
100	84.17 (5.15)	66.63 (1.36)	69.76 (2.16)	80.8 (2.84)
300	286.00	261.60 (18.59)	NT[d]	NT

[a]Mean Cu concentrations in test solutions used for renewal (n = 3, except for test solution of 300 μg/l).
[b]Mean Cu concentrations at the end of the renewal period (n = 5; n = 2 for test solution of 300 μg/l for Week 3).
[c]Values in parentheses are standard deviations.
[d]Not tested

TABLE II
Copper concentrations in microcosm test

Nominal Cu concentration (ug/l)	Concentration of added Cu (ug/l)[a]	Cu concentrations (ug/l)[b]		
		Week 1	Week 2	Week 3
0	< 5.00	< 5.00	< 5.00	< 5.00
3	< 5.00	< 5.00	< 5.00	< 5.00
10	9.63 (0.42)[c]	6.33 (0.55)	7.22 (0.62)	7.27 (0.59)
30	27.26 (1.35)	16.65 (0.65)	15.93 (0.53)	16.21 (0.79)
100	87.47 (7.09)	54.11 (6.04)	40.90 (3.10)	42.09 (3.59)
300	268.31	150.11 (13.62)	NT[d]	NT

[a]Mean Cu concentrations in test solutions used for renewal (n = 3, except for test solution of 300 μg/l).
[b]Mean Cu concentrations during microcosm test. Averages between the initial and final Cu concentrations were used in calculations (n = 4).
[c]Values in parentheses are standard deviations.
[d]Not tested

(Trollope and Evans, 1976) and dead organic material. Copper toxicity depends on the content of detritus and dead cells in the system (Erickson, 1972); high organic matter content in our microcosms (114 mg ash-free dry weight/l) facilitated binding of Cu, thus lowering concentration of dissolved Cu.

Effect of copper concentration on the length of survival in single-species test is shown on Fig. 1. All *Simocephalus* neonates exposed to 300 μg Cu/l were eliminated in the first 2 days of the experiment. Exposure to 100 μg Cu/l caused a 90% mortality after 3 weeks, compared to 30% in the control.

Total number of young produced during the test period was higher at low copper concentrations than in the control. At concentrations of 0, 3, 10, and 30 μg Cu/l, the numbers of neonates produced were 199, 205, 231, and 365, respectively. A similar stimulatory effect of Cu on another cladoceran (*Daphnia magna*) was previously observed by Winner and Farrell (1976). Up to concentrations of 60 μg/l, the mean brood sizes of *Daphnia* were significantly higher than in the control. In our experiment, only 52 neonates were produced at 100 μg/l, since most of the first-generation organisms died. Offspring was not produced at 300 μg Cu/l.

Simocephalus densities declined over time in all microcosms, regardless of the Cu concentration to which they were exposed (Fig. 2). Population densities in different microcosms were not significantly different in the beginning of the experiment. Exposure to nominal concentration of 300 μg Cu/l caused a rapid elimination of *Simocephalus*. In microcosms containing nominal concentration of 100 μg Cu/l, *Simocephalus* densities were significantly higher than in the control. Other Cu concentrations did not have an effect significantly different from that in the control.

Population density of *Simocephalus* in aquatic microcosms depends on the length of their survival and number of offspring they produce. A stimulated production of youngs at low copper concentrations (previously observed in single-species tests at concen-

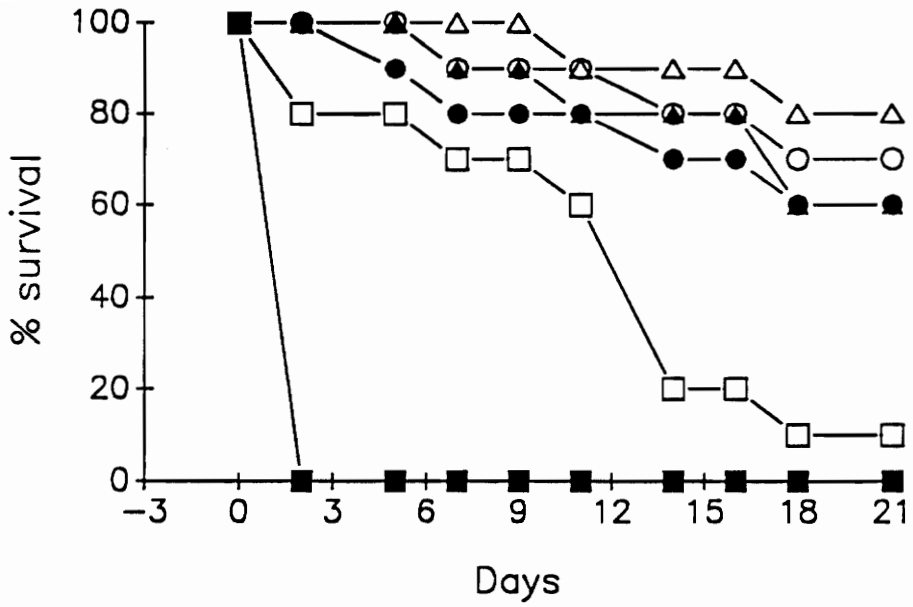

Fig. 1. Effect of copper on the length of *Simocephalus* survival in single-species test. Copper concentrations were: 0 μg/l (○); 3 μg/l (●); 10 μg/l (△); 30 μg/l (▲); 100 μg/l (□); 300 μg/l (■);

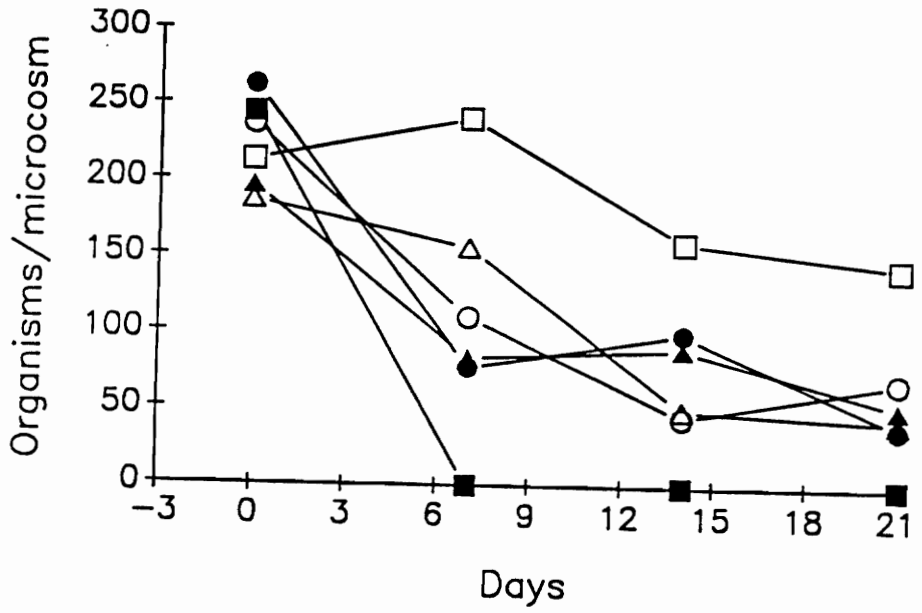

Fig. 2. Effect of copper on *Simocephalus* density in microcosm test. Copper concentrations were: 0 μg/l (○); 3 μg/l (●); 10 μg/l (△); 30 μg/l (▲); 100 μg/l (□); 300 μg/l (■);

trations up to 30 μg/l) was probably responsible for an increased *Simocephalus* density in microcosms with nominal concentration of 100 μg Cu/l, i.e., the measured concentration of 46 μg Cu/l. Similar increase in population density was documented by Marshall and Mellinger (1980) and Moore and Winner (1989), after exposure of cladocerans to low concentrations of heavy metals.

Table III shows the instantaneous rates of population growth (*r*). This statistic is considered to be useful as an index of the sensitivity to stress, since it integrates the effects on mortality and reproduction (Marshall, 1978; Winner et al., 1977). The highest *r*'s were observed at measured Cu concentrations of 30 μg/l in single-species tests and 46 μg/l in microcosm tests. Significant differences between *r*'s in copper-treated microcosms and the *r*'s in controls could not be detected, due to the high variability of final *Simocephalus* densities in replicate microcosms. The initial presence or absence of predatory *Chaoborus* larvae strongly influenced population dynamics of *Simocephalus* during the experiment (data not shown).

The responses of *Simocephalus* observed in single-species and microcosm tests after their exposure to similar Cu concentrations were very much alike: there was an increased neonate production at Cu concentrations of 30 to 46 μg/l; at concentrations above 100 μg/l there were no surviving organisms in either system. The results obtained in this experiment suggest the possibility of extrapolation from responses of isolated organisms in single-species tests to responses of that same species in microcosm tests; however, before futher validation, the results cannot be used to predict responses in natural ecosystems.

TABLE III

Instantaneous rates of population growth *(r)* in single-species and microcosm tests

Nominal Cu concentration	Single-species test	Microcosm test
0 ug/l	0.1317	-0.0604 (0.0363)[a]
3 ug/l	0.1371	-0.0896 (0.0536)
10 ug/l	0.1573	-0.0618 (0.0274)
30 ug/l	0.1846	-0.0597 (0.0283)
100 ug/l	0.1178	-0.0330 (0.0347)
300 ug/l	-[b]	-[b]

[a]Values in parentheses are standard deviations; n = 4
[b]No reproduction occurred at this concentration

LITERATURE CITED

Belanger, S. E., J. L. Farris, and D. S. Cherry. 1989. Effects of diet, water hardness, and population source on acute and chronic copper toxicity to *Ceriodaphnia dubia.* Arch. Environ. Contam. Toxicol. 18:601-611.

Cairns, J., Jr. 1988a. What constitutes field validation of predictions based on laboratory evidence?, p. 361-368. In: W. J. Adams, G. A. Chapman, and W. G. Landis (ed.), Aquatic Toxicology and Hazard Assessment: Tenth Volume, ASTM STP 971. American Society for Testing and Materials, Philadelphia, Pa.

Cairns, J., Jr. 1988b. Predicting, validating and monitoring effects of toxics on Large Lake ecosystems, p. 291-316. In: N. W. Schmidtke (ed.), Toxic Contamination in Large Lakes. Vol. II: Impact of Toxic Contaminants on Fisheries Management. Lewis Publishers, Chelsea, Mich.

Erickson, S. J. 1972. Toxicity of copper to a marine diatom in unenriched inshore seawater. J. Phycol. 8:318-323.

Harras, M. C., and F. B. Taub. 1985. Comparison of laboratory microcosms and field responses to copper, p. 57-74. In: T. P. Boyle (ed.), Validation and Predictability of Laboratory Methods for Assessing the Fate and Effects of Contaminants in Aquatic Ecosystems. American Society for Testing and Materials, Philadelphia, Pa.

Hodson, P. V., U. Borgmann, and H. Shear. 1979. Toxicity of copper to aquatic biota, p. 307-372. In: J. O. Nriagu (ed.), Copper in the Environment. John Wiley & Sons, New York.

Horning, W. B. and C. I. Weber. 1985. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. U.S. EPA, EPA/600?4-85/014, Cincinnati, Oh.

Kimball, K. D. and S. A. Levin. 1985. Limitations of laboratory bioassays: the need for ecosystem-level testing. BioScience 35:165-171.

Leffler, J. W. 1981. Aquatic microcosms and stress criteria for assessing environmental impact of organic chemicals. U. S. Environmental Protection Agency, National Technical Information Service, Springfield, Va.

Marshall, J. S. 1978. Field verification of cadmium toxicity to laboratory *Daphnia* populations. Bull. Environ. Contam. Toxicol. 20:387-393.

Marshall, J. S. and D. L. Mellinger. 1980. Dynamics of cadmium-stressed plankton communities. J. Fish. Res. Board Can. 37:403-414.

Moore, M. V. and Winner, R. W. 1989. Relative sensitivity of *Ceriodaphnia dubia* laboratory tests and pond communities of zooplankton and benthos to chronic copper stress. Aquat. Toxicol. 15_311-330.

Pontasch, K. W., B. R. Niederlehner, and J. Cairns, Jr. 1989. Comparisns of single-species, microcosm and field responses to a complex effluent. Environ. Toxicol. Chem. 8:521-532.

Roberts, J. R., D. W. Rodgers, J. R. Bailey, and M. A. Rorke. 1978. Polychlorinated biphenyls: biological criteria for an assessment of their effects on environmental quality. Associate Committee on Scientific Centers for Environmental Quality. National Research Council of Canada, Ottawa.

Trollope, D. R. and B. Evans. 1976. Concentrations of copper, iron, lead, nickel and zinc in freshwater algal blooms. Environ. Pollut. 11:109-116.

Winner, R. W. and Farrell, M. P. 1976. Acute and chronic toxicity of copper to four species of *Daphnia*. J. Fish. Res. Board Can. 33:1685-1691.

Winner, R. W., T. Keeling, R. Yeager, and M. P. Farrell 1977. Effect of food type on the acute and chronic toxicity of copper to *Daphnia magna*. Freshwater Biol. 7:343-349.

Summary of Results

Chapter 1

- The densities of genetically-engineered and wildtype *Erwinia carotovora* strains declined at the same rate in water and sediment of aquatic microcosms, falling in 32 days below the level of detection by viable counts.
- Inoculation with genetically or wildtype strain caused an increase in densities of total and proteolytic bacteria in water and sediment of aquatic microcosms.
- Inoculation of genetically-engineered or wildtype *E. carotovora* had no apparent effect on the densities of amylolytic and pectolytic bacteria in water and sediment.
- Genetically-engineered and wildtype *E. carotovora* did not have significantly different effects on the densities of specific functional groups of indigenous bacteria.

Chapter 2

- Enrichment media, antibiotic resistance, and most probable number (MPN) analysis can be used to enumerate as few as 1 to 10 target cells/10 g soil.
- Genetically-engineered *E. carotovora* introduced into soil microcosms declined at a rate of 1.2 log units/g soil/10 days and then disappeared.

Chapter 3

- Genetically-engineered *E. carotovora* persisted significantly longer in thermally-perturbed microcosms than in microcosms that did not undergo temperature stress.
- Inoculation with genetically-engineered *E. carotovora* did not affect the recovery of total and cellulolytic indigenous populations in perturbed microcosms.
- Recolonization of indigenous proteolytic bacteria seemed to be retarded in GEMs presence, possibly due to the competitive exclusion.

Chapter 4

- The effect of engineered and wildtype *E. carotovora* on densities of total bacteria, Actinomycetes, and *Pseudomonas* spp. was not significantly different.
- In a competition experiment, wildtype was not displaced by the engineered *E. carotovora*; both strains declined at a same rate in water and in sediment.
- Treatment with engineered bacteria did not change biomass values (ash-free dry weight) of the receiving community, but caused a transitory increase in its metabolic activity.
- In this study low risk was demonstrated; genetically-engineered *E. carotovora* did not persist, displace resident species, or affect the metabolic activity in aquatic microcosms.

Chapter 5

- Most of the copper added to aquatic microcosms was bound to sediment particles.
- In microcosms containing nominal concentrations of 100 μg Cu/kg sediment (about 0.5 mg/l of overlying water), chlorophyll a content and respiration significantly decreased compared to the control.

• In microcosms containing nominal concentration of 1000 mg Cu/kg (about 20 mg Cu/l of overlying water), the community showed a significant decrease in production, respiration, respiration/biomass ratio, ATP, and chlorophyll a; assimilation ratio and autotrophic index were significantly higher than in the control.

Chapter 6

• Both in single-species and microcosm tests, an increased production of youngs occurred after exposure of *Simocephalus exspinosus* to 30-46 μg Cu/l; at these copper concentrations, instantaneous rates of population growth were higher than in the controls.

• The organisms did not survive exposure to concentrations > 100 μg Cu/l in either test system.

• Responses of *Simocephalus* exposed to copper were similar in both test systems.

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B.S., Biology and Ecology, 1985, Faculty of Natural Sciences and Mathematics, University of Zagreb, Zagreb, Yugoslavia.
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Professional Employment

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Investigated impact of sediment-associated copper on structure and function of aquatic microcosms. Assessed persistence of genetically engineered *Erwinia carotovora* and investigated its effect on indigenous bacterial populations in aquatic microcosms. Developed a method for enumerating genetically-engineered microorganisms of low density in environmental samples. Participated in training for undergraduate honor students.

1987-88 Graduate Teaching Assistant, Department of Biology, VPI&SU.
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Publications

Scanferlato, V. S., D. R. Orvos, G. H. Lacy, and J. Cairns, Jr. Enumerating low densities of genetically-engineered *Erwinia carotovora* in soil. Let. Appl. Microbiol. (in press)

Stromberg, V. K., D. R. Orvos, V. S. Scanferlato, G. H. Lacy, and J. Cairns, Jr. *In planta* competition among cell-degrading enzyme mutants and wildtype strains of *Erwinia carotovora,* Published symposium, *In:* Proc. 7th Internatl. Conf. on Plant Pathogenic Bacteria, 11-16 June 1989, Hun. Acad. Sci., Budapest (in press)

Habdija, I., V. Sostarec, B. Primc and D. Durut-Beslac. Influence of ecological parameters on community structure, biomass, and trophic relations of zooplankton in Plitvice Lakes, Yugoslavia. Periodicum Biologorum (in press)

Scanferlato, S. V., D. R. Orvos, J. Cairns, Jr., and G. H. Lacy. Genetically-engineered *Erwinia carotovora* in aquatic microcosms: survival and effects on functional groups of indigenous bacteria. Appl. Environ. Microbiol. 55:1477-1482.

Sostarec, V. 1987. Die Zusammensetzung des Crustaceenplanktons in dem neben der Sava gelegnen Baggersee Jarun. 26. Arbeitstagung der IAD. -Wissenschaftliche Kurzreferate, 207-209.

Mestrov, M., I. Habdija, B. Stilinovic, V. Tavcar, R. Lattinger, M. Kerovec, Z. Maloseja, B. Primc, and V. Sostarec, 1987. Der Einfluss des ufernahen Grundwassers auf die Biozonotischen and Trophischen Eigenschaften des an der Sava gelegnen Baggersees Jarun. 26. Arbeitstagung der IAD. Wissenschaftliche Kurzereferate, 156-160.

Abstracts / Presented Papers

Orvos, D. R., V. S. Scanferlato, G. H. Lacy, and J. Cairns, Jr. Fate and effects of genetically-engineered *Erwinia carotovora* in terrestrial and aquatic microcosms. 89th Annual Meeting of the American Society for Microbiology, New Orleans, May 1989.

Sostarec, V., D. Orvos, J. Cairns, Jr. and G.H. Lacy. Persistence of genetically engineered *Erwinia carotovora* in aquatic microcosms. Ninth Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC), Arlington, November 1988.

Sostarec, V. Seasonal dynamics of cladoceran and copepod populations of Lake Jarun, Yugoslavia, in relation to ecological factors. Annual Meeting of the Virginia Academy of Science, Charlottesville, May 1988.

Sostarec, V. Cladocera and Copepoda as indicators of the trophic state of Lake Jarun. Third congress of Croatian biologists, Mali Losinj, October, 1987.

Mestrov, M., I. Habdija, B. Stilinovic, B. Primc, M. Kerovec, and V. Sostarec. Impact of ground water on biocenoses and trophic characteristics of Lake Jarun. Sava River Simposium. Zagreb, November 1987.

Sostarec, V. and M. Kerovec. Effect of chlorine on periphyton community under laboratory conditions. Second congress of Croatian biologists, Zadar, October 1984.

Grants Received

Sigma Xi, Grants-in-Aid of Research.
Comparison of persistence of genetically engineered microorganism *Erwinia carotovora* in ecologically perturbed and non-perturbed systems.

Graduate Student Assembly Graduate Research Development Project.
Enumeration of genetically-engineered *Erwinia carotovora* of low density in soil samples.

Honors and Awards

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1984. "Majska nagrada", award for the best student paper. Title: "Determination of air pollution zones in Kutina using lichens as indicators"

Professional Societies

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