Arch Microbiol (1988) 150:320–325

Archives of
Microbiology

Factors affecting the survival and growth of bacteria introduced into lake water

Phillip R. Scheuerman*, John P. Schmidt, and Martin Alexander

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, NY 14853, USA

Abstract. The populations of *Pseudomonas* sp. B4, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus flavus*, and *Rhizobium leguminosarum* biovar *phaseoli* declined rapidly in lake water. The initially rapid decline of the two pseudomonads and *R. phaseoli* was followed by a period of slow loss of viability, but viable cells of the other species were not found after 10 days. The rapid initial phase of decline was not a result of *Bdellovibrio* spp., bacteriophages, or toxins in the water since *Bdellovibrio* spp. were not present and passage of the lake water through filters that should not have removed bacteriophages or soluble toxins led to the elimination of the rapid phase of decline. The addition of 250 μg of cycloheximide and 30 μg of nystatin per ml eliminated viable protozoa form the lake water, and the population of *Pseudomonas* sp. B4 did not fall and the decline of *E. coli* and *K. pneumoniae* was delayed or slowed under these conditions. *Pseudomonas* sp. L2 proliferated rapidly in lake water amended with glucose, phosphate, and NH_4NO_3, but its numbers subsequently fell abruptly; however, in water amended with cycloheximide and nystatin, which killed indigenous protozoa, the population density was higher and the fall in numbers was delayed. Of the nutrients, the chief response was to carbon, but when glucose was added, phosphorus and nitrogen stimulated growth further. Removing other bacteria by filtering the lake water before inoculation with *Pseudomonas* sp. L2 suggested that competition reduced the extent of response of the pseudomonad to added nutrients. We suggest that the decline in lake water of bacteria that are resistant to starvation may be a result of protozoan grazing and that the extent of growth of introduced species may be limited by the supply of available carbon and sometimes of nitrogen and phosphorus, and by predation by indigenous protozoa.

Key words: *Escherichia* – *Klebsiella* – Lake water – *Micrococcus* – Multiplication – Protozoa – *Pseudomonas* – *Rhizobium* – Survival

The factors affecting survival and growth of bacteria in natural waters are not well understood. Interest in these factors has increased, however, because of concern with the release of genetically engineered organisms into natural ecosystems. Earlier studies of factors affecting bacterial survival have usually dealt with pathogens or indicator species of public health significance, although more recent investigations have been concerned with other species.

Several factors have been suggested to have a significant impact on the survival of bacteria in natural waters. Chamberlain and Mitchell (1978) proposed that light was a significant stress affecting bacterial survival in marine environments, and the susceptibility of *Escherichia coli* to photoinactivation has been shown (Lessard and Sieburth 1983). Temperature is also known to influence the persistence of *E. coli* in natural waters (McFeters and Stuart 1972), and the lack of persistence of some species has been attributed to the presence in water of low-molecular-weight toxins (Klein and Alexander 1986). Evidence is also accumulating that small zooplankton may be active in grazing on bacteria in freshwater environments (Rieman 1985; Sanders and Porter 1986). Bacteria introduced into the same waters may differ markedly in their persistence (Liang et al. 1982), and for some species at least, these differences appear to be attributable to their resistance to starvation (Sinclair and Alexander 1984). Some marine vibrios even maintain their viability in salts solution for as long as one year (Novitsky and Morita 1977). Little information is available on the factors affecting the growth of individual bacterial species in natural waters (Alexander 1986).

It is not yet possible to make convincing predictions of the ability of as yet untested bacteria to survive or multiply in natural waters. Without such predictive ability, it is difficult to assess the potential problems and risks associated with the release of genetically engineered microorganisms into natural environments. Therefore, a study was initiated to characterize some of the factors that affect the ability of bacteria to survive and multiply in lake water.

Materials and methods

Erlenmeyer flasks (250 ml) were washed for a minimum of 2 h in No-chromix (Godax Laboratories, New York, NY, USA), a metal-free oxidizing agent, and subsequently rinsed three times each in tap water and then distilled water. The flasks were closed with foam plugs, which were then covered with foil, and the flasks were autoclaved for 30 min. Flasks containing 100 ml of lake water were incubated in the dark

**Present address:* Dept. of Environmental Health, School of Public and Allied Health, East Tennessee State University, Johnson City, TN 37614, USA

Offprint requests to: M. Alexander

at 30° C on a rotary shaker operating at 100 rpm. Water samples, which were obtained from Beebe Lake, Ithaca, NY, USA, were used within 1 h of collection. The pH of different lake water samples ranged from 7.4 to 8.0.

Cultures of *Escherichia coli* C-3000, *Micrococcus flavus, Klebsiella pneumoniae* KNO; *Pseudomonas* sp. B4, and *Pseudomonas* sp. L2 were grown in half-strength Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA). *Rhizobium leguminosarum* biovar *phaseoli* was grown in yeast extract-mannitol (YEM) broth (Sinclair and Alexander 1984). The media also contained the antibiotics indicated below. The cultures were incubated at 30° C until they reached the late logarithmic phase, and the cells then were collected by centrifugation at $8{,}000 \times g$ for 10 min at 4° C and washed three times with either autoclaved or filter-sterilized lake water for experiments involving autoclaved or filter-sterilized lake waters, respectively. The cells were then inoculated into the lake water to a density of 10^4 to 10^6 CFU/ml.

Bacteria were enumerated using the drop-plate technique (Hoben and Somasegaran 1982) on yeast extract-mannitol agar containing 1.0 mg of streptomycin and 50 μg of erythromycin per ml for *R. phaseoli,* and Trypticase soy agar (composed of 15 g of Trypticase soy broth, 1.25 g each of K_2HPO_4 and KH_2PO_4, and 15 g of agar per liter of distilled water) amended with 1.0 mg of streptomycin and 50 μg of erythromycin per ml for *Pseudomonas* sp. L2, 1.0 mg of streptomycin, 50 μg of erythromycin, and 10 μg of brilliant green per ml for *Pseudomonas* sp. B4, and 1.0 mg of spectinomycin and 100 μg of erythromycin per ml for *M. flavus*. These media were also supplemented with 250 μg of cycloheximide per ml. *E. coli* was counted on eosin methylene blue agar (Difco) without antibiotics.

The survival of bacteria was tested in lake water that was untreated, autoclaved for 30 min, or sterilized by passage through a series of membrane filters with pore sizes of 8.0, 0.45, and 0.2 μm (Gelman Sciences, Ann Arbor, MI, USA). Triplicate flasks were incubated in the dark at 30° C on a rotary shaker operating at 100 rpm, and the lake water samples were assayed for the test bacterium at various intervals using triplicate plates at each dilution.

In studies of bacterial growth, nutrients were added to the lake water to give final concentrations of 1 mg of glucose, 0.14 mg of K_2HPO_4, and 0.29 mg of NH_4NO_3 per ml. All other conditions were as described for the survival experiments. In some instances, 250 μg of cycloheximide and 30 μg of nystatin per ml were added to inhibit eucaryotes. The trophic forms of protozoa were counted microscopically. The possible presence of *Bdellovibrio* spp. was determined by the double-layer procedure with *E. coli* or *Pseudomonas* sp. L2 as the test bacterium and trypticase soy agar, except that 7.5 g of agar per liter was used for the overlay medium.

Results

Tests were conducted of the survival of members of five genera in nonsterile and sterile lake water. The test bacteria were added at concentrations (10^4 to 10^7 cells/ml) equal to or greater than the densities of indigenous bacteria (10^4 to 10^6 cells/ml). *Pseudomonas* sp. L2 survived with little change in cell density in both autoclaved and filter-sterilized lake water, and *R. phaseoli, Pseudomonas* sp. B4, *E. coli, K. pneumoniae,* and *M. flavus* slowly declined in abundance in both autoclaved and filter-sterilized lake water, sometimes after a brief period of multiplication (Fig. 1). In contrast, the populations of each organism declined markedly in nonsterile lake water. The rate of decline of *R. phaseoli* and the two pseudomonads diminished with time. Although viable cells of *E. coli, K. pneumoniae,* and *M. flavus* were detected at 5 days, none was found at 10 days. *Bdellovibrio* spp. acting on *Pseudomonas* sp. L2 or *E. coli* were not detected in the lake water.

To test for the formation of toxic products during incubation, freshly collected lake water was incubated at 30° C in the dark for 2 days on a rotary shaker operating at 100 rpm. Some of the water samples were then autoclaved or sterilized by filtration, and the sterile and nonsterile water samples were inoculated with *K. pneumoniae* or *Pseudomonas* sp. L2. Under these conditions, the pattern of survival of both bacteria were essentially the same as in waters that had not been first incubated in the laboratory (data not shown).

To determine the possible role of protozoa in the decline in nonsterile lake water, 250 μg of cycloheximide and 30 μg of nystatin per ml were added to half of the samples, and four of the organisms were added to both unamended and antibiotic-amended waters. The numbers of active protozoa remained constant at 10^4 to 10^5 cells per ml in untreated samples. In inhibitor-amended lake water inoculated with the test bacteria, about 100 protozoa were detected per ml at the end of 1 day, but none was detected thereafter. Except at the end of 1 day, *Pseudomonas* sp. L2 did not show any appreciable difference in survival in the presence or absence of protozoa (Fig. 2). However, the cell densities of *Pseudomonas* sp. B4, *E. coli* and *K. pneumoniae* were higher in the inhibitor-amended waters, in which the protozoa were markedly suppressed. Nevertheless, the populations of the last two species did decline in the inhibitor-treated water, the rate being especially marked in the last 2 days. Protozoa, especilly microflagellates, were at population densities of 10^4 to 10^5 cells per ml in the waters not receiving the eucaryotic inhibitors. It is noteworthy that the pattern of *Pseudomonas* sp. L2 survival was different in this test from that noted previously. The differences may have resulted from dissimilar bacterial communities in these water samples, which were collected during the winter, from those present during the summer and autumn in the samles used previously. Addition of the inhibitors to water not inoculated with any of the test species resulted in an increase in numbers of indigenous bacteria, but no such increase was evident in untreated water samples (data not shown).

In studies designed to determine why *Pseudomonas* sp. L2 did not grow in nonsterile lake water, it was assumed that the chief constraints were lack of an adequate supply of readily available C, too low concentrations of N or P, or predation by protozoa. Hence, lake water was amended with: (i) 1.0 mg of glucose, 0.14 mg of K_2HPO_4, 0.29 mg of NH_4NO_3, 250 μg of cycloheximide and 30 μg of nystatin per ml; (ii) P, N, and the eucaryotic inhibitors; (iii) glucose, N, and P; (iv) glucose and the inhibitors; or (v) nothing. These treatments may be viewed as (i) a possibly complete supplement to overcome all primary constraints, (ii) complete minus C, (iii) complete minus inhibitors, and (iv) complete minus N and P, and (v) unsupplemented. One sample of water received no amendments, and all were inoculated with *Pseudomonas* sp. L2. The addition of glucose, N, and

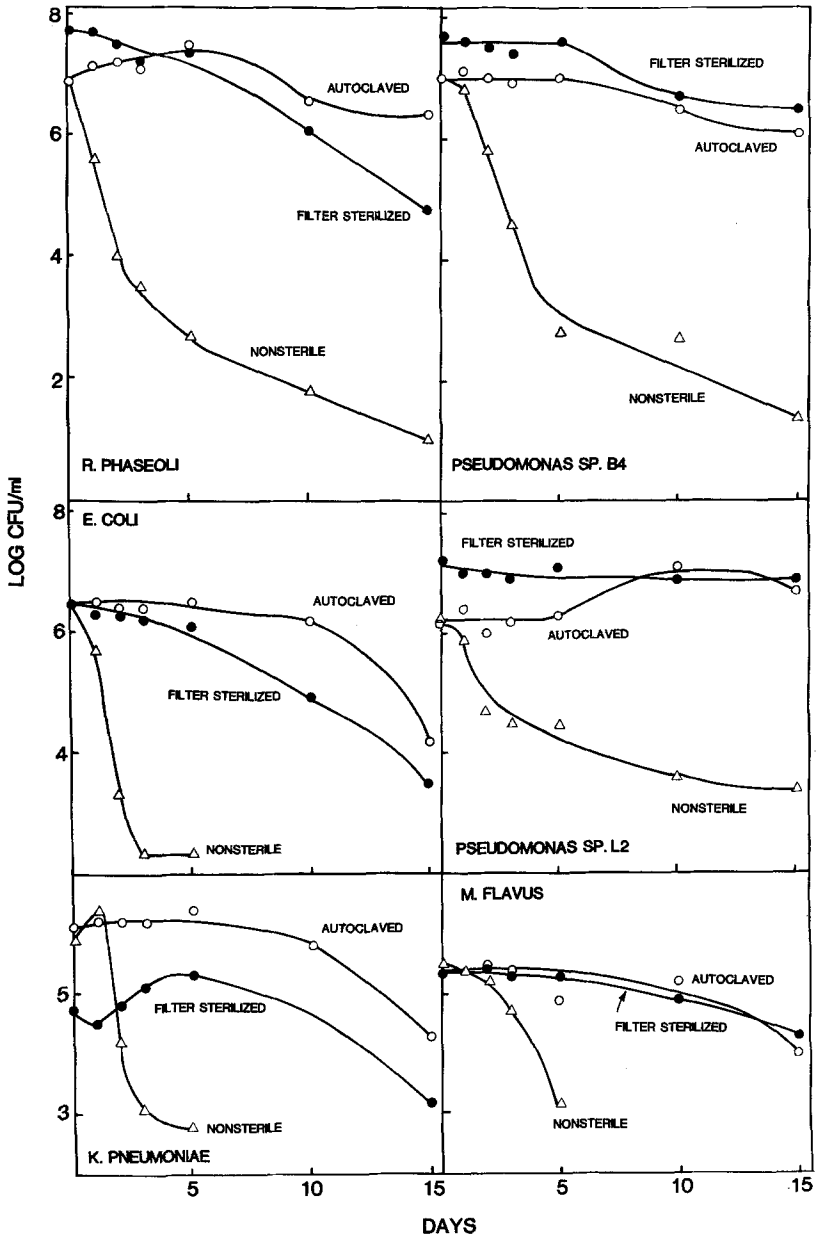

Fig. 1
Survival of *R. phaseoli*, *Pseudomonas* sp. B4, *E. coli*, *Pseudomonas* sp. L2, *K. pneumoniae* and *M. flavus* in nonsterile lake water and in lake water sterilized by autoclaving or filtration

P together with the eucaryotic inhibitors resulted in extensive multiplication of the test bacterium, and a population density of about 10^{10} per ml was reached at 3 days, after which the cell numbers declined (Fig. 3 bottom). When glucose, N, and P but no eucaryotic inhibitors (treatment C, N, P) were added or when glucose and the inhibitors but no N and P (treatment C, inhibitors) were added, an increase in *Pseudomonas* sp. L2 density was evident, but it was not as marked; moreover, the decline began sooner than when all constraints were removed. On the other hand, when no organic nutrient was added, even though N and P were in adequate supply and eucaryotes were suppressed (treatment N, P, inhibitors), the organism did not multiply.

To determine the possible contribution of competition to limiting the growth of *Pseudomonas* sp. L2, samples of lake water were filter sterilized and amended with (i) glucose, K_2HPO_4, and NH_4NO_3 at the same concentrations as above (treatment C, N, P), (ii) glucose alone (treatment C), (iii) N and P only (treatment N, P), or (iv) nothing. The samples were then inoculated with *Pseudomonas* sp. L2. Under these conditions, in which no protozoa or competing bacteria were present, the addition of C caused only a slight increase in abundance of *Pseudomonas* sp. L2 (Fig. 3 top). Supplementation with N and P not only did not enhance growth but caused a slight decline in bacterial numbers. However, the addition of C, N, and P markedly enhanced growth, although the population size declined after 2 days.

A further test of the possible role of competition and predation was performed with a smaller inoculum of *Pseudomonas* sp. L2. One water sample was amended with (i) the nutrients and inhibitors at the concentrations previously used, and filter-sterilized samples were amended with (ii) inhibitors only, (iii) nutrients only, or (iv) nutrients and inhibitors. *Pseudomonas* sp. L2 grew in the nutrient-supplemented lake water containing eucaryotic inhibitors, but the extent of growth was small (Fig. 4). Presumably the indigenous bacteria readily used much of the available nutrients because of the absence of grazing pressure. More extensive multiplication was noted following inoculation of the sterile, inhibitor-amended water, the rapid development

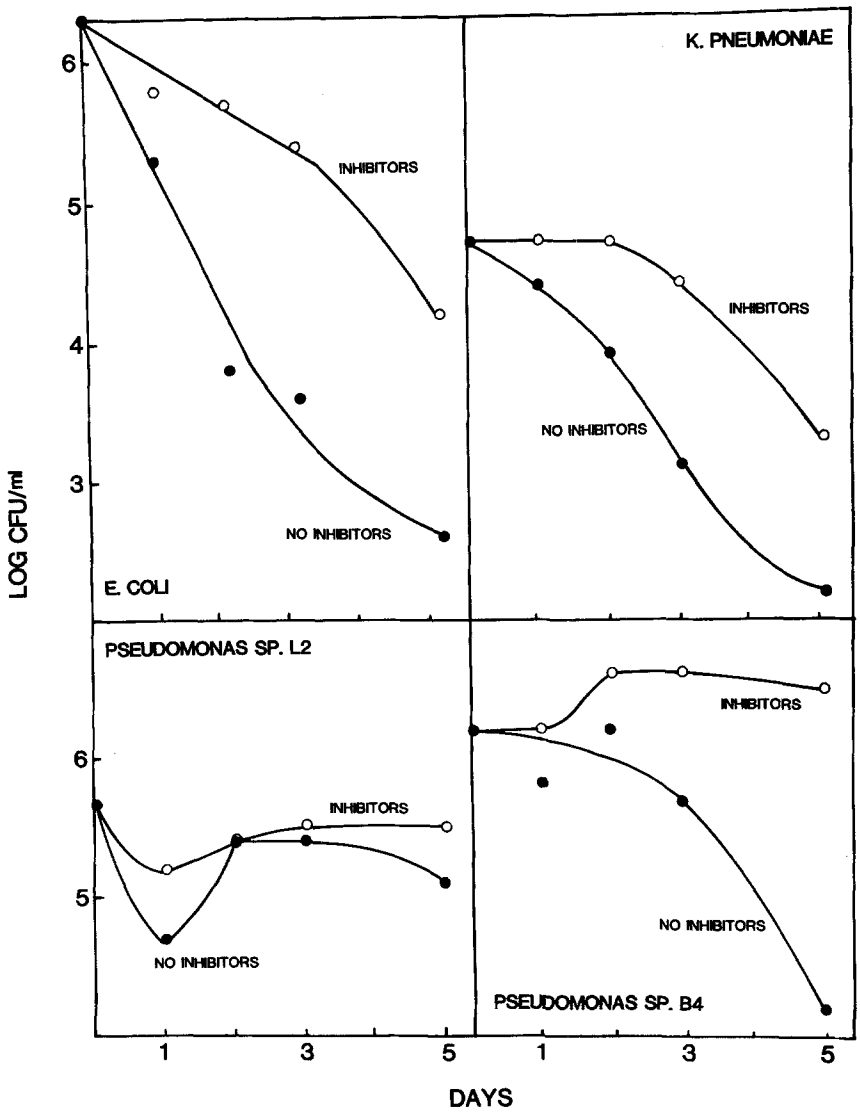

Fig. 2
The response of *E. coli*, *K. pneumoniae*, *Pseudomonas* sp. L2, and *Pseudomonas* sp. B4 to the addition of eucaryotic inhibitors to lake water

under these circumstances showing that cycloheximide and nystatin had little effect on the bacterium. The addition of nutrients, however, further enhanced bacterial development.

Discussion

Two survival patterns were observed. The first was a rapid decline followed by a period of slow or no loss of viability. The second was a decline to levels below detection limits within the first 5 to 10 days. These results are similar to those of Liang et al. (1982). However, they observed that *K. pneumoniae* died rapidly in both sterile and nonsterile lake water, but no such rapid decline in sterile lake water was noted in the present study. The differences could result from the presence of toxins in the water in the earlier study, such toxins having been found in lake water samples (Klein and Alexander 1986).

The poor survival of the test species in nonsterile lake water was not a result of the existence of toxins in the freshly sampled lake water, because the organisms persisted in samples that had been passed through 0.2-μm pore-size filters. Such filters should have allowed passage not only of toxins but also of bacteriophages that might have been present in the lake water; hence, because of the survival in the filtered water samples, bacteriophages account for little or none of the decline. *Bdellovibrio* spp. were not present in these waters, so they also have no role in the reduction in population size. Toxins formed during the incubation were also not responsible for the decline since incubation of the lake water for 2 days before adding *K. pneumoniae* or *Pseudomonas* sp. L2 did not change the pattern of survival. Toxins, bacteriophages, or *Bdellovibrio* may be important in the decline of other species or in other aquatic environments.

On the other hand, protozoa apparently are involved in the elimination of many of the cells of the test organisms. A role for protozoa is evident by the increased survival in lake water amended with eucaryotic inhibitors, a treatment that eliminated these predators. Even when growth of the bacteria was enhanced by the addition of high concentrations of nutrients, an impact of predation was noted since the addition of eucaryotic inhibitors to the lake water allowed for greater cell numbers. An especially marked effect of grazing on *Pseudomonas* sp. L2 was evident when nutrients were added to the lake water, probably because its density increased to levels above the threshold cell density for predation by protozoa (Alexander 1981). The observation that several bacterial species were suppressed by protozoa even when the cell densities of the test bacteria were below the

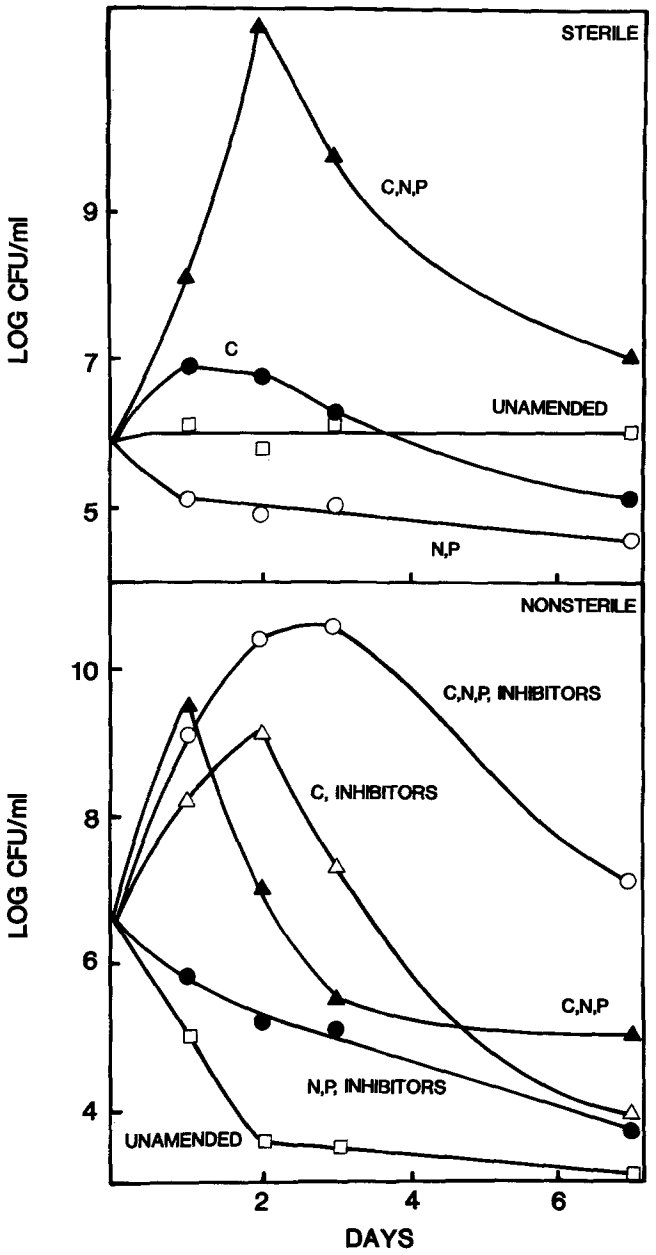

Fig. 3. The effect of the addition of mixtures of glucose, K_2HPO_4, NH_4NO_3, and eucaryotic inhibitors on the population of *Pseudomonas* sp. L2 added to nonsterile (*bottom*) and filter-sterilized (*top*) lake water

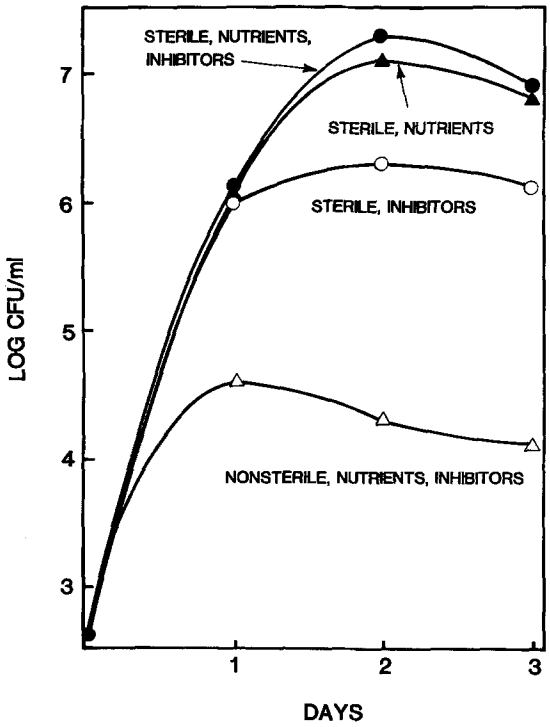

Fig. 4. Growth of *Pseudomonas* sp. L2 added to nonsterile lake water amended with cycloheximide, nystatin, glucose, K_2HPO_4, and NH_4NO_3 or sterile lake water amended with the inhibitors, the nutrients, or both

threshold suggests that they were consumed as the protozoa were grazing on alternative prey (Mallory et al. 1983). The lack of suppression of *Pseudomonas* sp. L2 during the grazing on other bacterial prey could be a consequence of growth of the pseudomonad at a rate about the same as the grazing rate; however, when alternative prey are at levels above the threshold for grazing, the density of the pseudomonad can be reduced if its multiplication rate is less than the grazing rate. It has been reported that *E. coli* is eliminated by protozoa in estuarine waters (Enzinger and Cooper 1976; McCambridge and McMeekin 1980).

Sanders and Porter (1986) noted that cycloheximide did not eliminate all the protozoa from a sample of eutrophic lake water. The direct microscopic counts failed to show active protozoa in the waters treated with both cycloheximide and nystatin, whereas the density of protozoa in the untreated waters was between 10^4 to 10^5 organisms per ml. Therefore, the addition of both inhibitors is an effective means of suppressing protozoa in lake water. Tremaine and Mills (1987) noted that cycloheximide inhibited anaerobic bacteria, but such inhibitions were not evident in the present study as evidenced by the similar rates and extents of growth of *Pseudomonas* sp. L2 in glucose-amended sterile lake water with and without the inhibitors.

Studies of the constraints on growth of *Pseudomonas* sp. L2 show that a major limitation to the multiplication of an introduced species is the paucity of available carbon. Without a supply of organic nutrients, such an organism will not increase in abundance. However, the introduced species must compete for the limiting nutrients with the indigenous populations, so that only a portion of the supply of nutrients will be available to it; once the supply is exhausted by the competitors, the introduced population will not multiply further. The data further show that a deficiency of N and P appears if there is abundant available organic C, and this may affect the final population sizes of the indigenous and the allochthonous bacteria. However, it is not clear whether sufficient available C will be present for heterotrophs in natural waters for N or P deficiency to be a significant constraint for the replication of an alien bacterium.

Eucaryotes, presumably protozoa, are also major constraints on the growth of bacteria in waters that receive large inputs of organic carbon, and these grazers may have a dramatic impact on their prey. Inasmuch as intense predation presumably requires the presence of a bacterial community with more than about 10^6 cells per ml (Alexander

1981), which in turn requires an adequate supply of organic carbon, protozoa are likely to have an especially pronounced impact at sewage outfalls, in the vicinity of excretions from phytoplankton blooms, or under circumstances where there are continuous episodic influxes of organic matter into aquatic ecosystems. Recent studies add to the body of information that freshwater (Findlay et al. 1986) and marine protozoa (Rassoulzadegan and Sheldon 1986; Sherr and Sherr 1987) are important in controlling bacterial populations, even in oligotrophic waters.

It is surprising that the counts of *Pseudomonas* sp. L2 in nutrient-supplemented lake water were in the vicinity of 10^{11} per ml in one instance. These high numbers could result from experimental error or from the development of small cells of the test bacterium. Although no attempt was made to determine cell size in this study, which involved replicated counts that agreed closely, similarly high counts of *Corynebacterium* sp. in soil amended with 1.0% glucose have been observed (M. J. Acea-Escrich and M. Alexander, unpublished data).

Although the present findings are from studies performed under artificial conditions, they do suggest some of the factors that may affect the survival and multiplication of genetically engineered or other allochthonous bacteria in aquatic environments. Few species and few water samples have been investigated. Therefore, additional inquiry is warranted to define more fully the biotic and abiotic factors that determine the capacity of bacteria to persist and to multiply in aquatic ecosystems. Future studies should deal with more natural conditions. Correlation of those new studies, field tests and the approaches used herein should facilitate the development of more useful procedures for evaluating the behavior of genetically altered microorganisms.

Acknowledgements. We thank Joan Ellis for excellent technical assistance and A. W. Bourquin and H. P. Pritchard for advice. This investigation was supported by US Environmental Protection Agency cooperative agreement CR8122331010. The paper has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred.

References

Alexander M (1981) Why microbial predators and parasites do not eliminate their prey and hosts. Annu Rev Microbiol 35:113–133

Alexander M (1986) Survival and growth of bacteria. Environ Manag 10:464–469

Chamberlin CE, Mitchell R (1978) A decay model for enteric bacteria in natural waters. In: Mitchell R (ed) Water pollution microbiology, vol 2. John Wiley, New York, pp 325–348

Enzinger RM, Cooper RC (1976) Role of bacteria and protozoa in the removal of *Escherichia coli* from estuarine waters. Appl Environ Microbiol 31:758–763

Findlay S, Carlough L, Crocker MT, Gill HK, Meyer JL, Smith PH (1986) Bacterial growth on macrophyte leachate and fate of bacterial production. Limnol Oceanogr 31:1335–1341

Hoben HJ, Somasegaran P (1982) Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl Environ Microbiol 44:1246–1247

Klein TM, Alexander M (1986) Bacterial inhibitors in lake water. Appl Environ Microbiol 52:114–118

Lessard EJ, Sieburth JMcN (1983) Survival of natural sewage populations of enteric bacteria in diffusion and batch chambers in the marine environment. Appl Environ Microbiol 45:950–959

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

Mallory LM, Yuk CS, Liang LN, Alexander M (1983) Alternative prey: a mechanism for elimination of bacterial species by protozoa. Appl Environ Microbiol 46:1073–1079

McCambridge J, McMeekin TA (1980) Relative effects of bacterial and protozoan predators on survival of *Escherichia coli* in estuarine water samples. Appl Environ Microbiol 40:907–911

McFeters GA, Stuart DG (1972) Survival of coliform bacteria in natural waters: fieled and laboratory studies with membrane-filter chambers. Appl Microbiol 24:805–811

Novitsky JA, Morita RY (1977) Survival of a psychrophilic marine vibrio under long-term nutrient starvation. Appl Environ Microbiol 33:635–641

Rassoulzadegan F, Sheldon RW (1986) Predator-prey interactions of nanozooplankton and bacteria in an oligotrophic marine environment. Limnol Oceanogr 31:1010–1021

Riemann B (1985) Potential importance of fish predation and zooplankton grazing on natural populations of freshwater bacteria. Appl Environ Microbiol 50:187–193

Sanders RW, Porter KG (1986) Use of metabolic inhibitors to estimate protozooplankton grazing and bacterial production in a monomictic eutrophic lake with an anaerobic hypolimnion. Appl Environ Microbiol 52:101–107

Sherr EB, Sherr BF (1987) High rates of consumption of bacteria by pelagic ciliates. Nature (Lond)) 325:710–711

Sinclair JL, Alexander M (1984) Role of resistance to starvation in bacterial survival in sewage and lake water. Appl Environ Microbiol 48:410–415

Tremaine SC, Mills AL (1987) Inadequacy of the eucaryote inhibitor cycloheximide in studies of protozoan grazing on bacteria at the fresh-water-sediment interface. Appl Environ Microbiol 53:1969–1972

Received November 22, 1987/Accepted March 28, 1988