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Impact of a Genetically Engineered Bacterium with Enhanced Alkaline Phosphatase Activity on Marine Phytoplankton Communities

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An indigenous marine *Achromobacter* sp. was isolated from coastal Georgia seawater and modified in the laboratory by introduction of a plasmid with a *phoA* hybrid gene that directed constitutive overproduction of alkaline phosphatase. The effects of this "indigenous" genetically engineered microorganism (GEM) on phosphorus cycling were determined in seawater microcosms following the addition of a model dissolved organic phosphorus compound, glycerol 3-phosphate, at a concentration of 1 or 10 μM. Within 48 h, a 2- to 10-fold increase in the concentration of inorganic phosphate occurred in microcosms containing the GEM (added at an initial density equivalent to 8% of the total bacterial population) relative to controls containing only natural microbial populations, natural populations with the unmodified *Achromobacter* sp., or natural populations with the *Achromobacter* sp. containing the plasmid but not the *phoA* gene. Secondary effects of the GEM on the phytoplankton community were observed after several days, evident as sustained increases in phytoplankton biomass (up to 14-fold) over that in controls. Even in the absence of added glycerol 3-phosphate, a numerically stable GEM population (averaging 3 to 5% of culturable bacteria) was established within 2 to 3 weeks of introduction into seawater. Moreover, alkaline phosphatase activity in microcosms with the GEM was substantially higher than that in controls for up to 25 days, and microcosms containing the GEM maintained the potential for net phosphate accumulation above control levels for longer than 1 month.

Valid questions can be raised about the potential ecological effects of genetically engineered microorganisms released deliberately or accidentally into natural environments. Such concerns once were countered with the argument that recombinant organisms would have reduced fitness for survival and growth in the environment due to increased metabolic load imposed by maintenance and expression of the foreign genes (6). However, experimental work is inconsistent with this argument; recent studies demonstrate that GEMs can survive for prolonged periods in the presence of natural microbial populations (20, 22, 37, 35).

Establishment of novel genetic material in natural ecosystems may occur either directly or indirectly. An introduced organism may establish a self-sustaining population within the natural bacterial community, possibly displacing indigenous species (7, 10). Alternatively, indirect establishment can occur when a novel DNA sequence is transferred to members of the native microbial assemblage (15). This latter pathway requires only that the engineered organism persist long enough for its DNA to be incorporated by an indigenous bacterium.

Current concern over the ecological consequences of releasing GEMs centers on the possibility that they could adversely effect ecosystem structure or function by altering biogeochemical processes, since perturbations to carbon and nutrient cycles could potentially bring about far-reaching, likely negative, impacts (30, 34), yet there have been relatively few studies on ecosystem-level effects of GEMs. Of the few reported, most have focused on microbial populations and processes in soil, and most have found no persistent measurable ecosystem-level effects (9, 16, 21, 26, 31, 36).

The present study uses a model native bacterium which has acquired an engineered trait to examine potential ecosystem-level effects on a planktonic marine ecosystem. We reasoned that if engineered genetic material were to become established in a natural ecosystem, this would most likely occur either by an indigenous organism's acquiring a gene from a released exotic strain or through the reintroduction of an indigenous organism to a natural system following genetic alteration (e.g., for bioremediation purposes). We modified an *Achromobacter* strain isolated from coastal seawater by introduction of a plasmid with a *phoA* hybrid gene that directed constitutive overproduction of alkaline phosphatase. As this modification enhanced the bacterium's ability to hydrolyze dissolved organophosphorus (DOP) compounds, the impacts of the model GEM on phosphorus cycling and microbial community structure were studied in seawater microcosms.

MATERIALS AND METHODS

Isolation of *Achromobacter* sp. strain B1. Water (500 ml) collected from a tidal creek at the University of Georgia Marine Institute on Sapelo Island, Ga., was amended with 0.05% sodium vanillate and incubated for 4 to 7 days (30°C, 200 rpm). Subsamples were streaked onto agar plates of half-strength basal salts minimal medium (29) with sea salts (40 g/liter; Sigma Chemical Co., St. Louis, Mo.) and 0.05% vanillate. Isolates were restreaked twice to ensure purity. One of these isolates was identified by fatty acid analysis (Microbial ID, Inc., Newark, Del.) as an *Achromobacter* sp. and used in further studies. This bacterium (*Achromobacter* sp. strain B1) grows on vanillate or cellobiose as a sole carbon and energy source, requires sodium ion (>2%) for optimal growth (as per the method of Baumann et al. [4]), and is naturally resistant to nalidixic acid (300 μg ml^{-1}). From these phenotypic characteristics, we developed a selective medium for *Achromobacter* sp. strain B1, VSA medium, consisting of 0.05% vanillate, 25 mM sodium-potassium phosphate (pH 7.0), 7 mM $(NH_4)_2SO_4$, 0.4 mM $MgCl_2$, 1 μM $FeSO_4$, 4 μM Na_2MoO_4, 2.5 μM $MnCl_2$, and 40 g of sea salts per liter.

Construction and analysis of genetically engineered *Achromobacter* strains. We selected spontaneous mutants of *Achromobacter* sp. strain B1 that were resistant to either streptomycin (500 μg ml^{-1}) or rifampicin (75 μg ml^{-1}) (Table 1) by plating on YTSS plates (1% yeast extract, 0.5% tryptone, 3% sea salts, and 2% agar) containing the indicated amounts of antibiotic. Broad-host-range plasmids pRK404 and pJH123 were transferred from *Escherichia coli* to streptomycin- or rifampicin-resistant *Achromobacter* strains, respectively, as described previously (27). Briefly, 5×10^7 log-phase cells each of the donor, recipient, and

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TABLE 1. Characteristics of *Achromobacter* strains

Strain	Plasmid	Phenotype[a]	PhoA activity[b]
B1	None	Wild type; Nal^r	0.8
B1.1	pRK404	Nal^r Str^r Tc^r	0.9
B1.2	pJH123	Nal^r Rif^r Tc^r; constitutively expressing alkaline phosphatase	36.0

[a] Nal^r, Rif^r, Str^r, and Tc^r, resistance to nalidixic acid, rifampicin, streptomycin, and tetracycline, respectively.

[b] Alkaline phosphatase activity is in nanomoles of *p*-nitrophenylphosphate hydrolyzed per minute per 10^9 cells, measured as described by Huang and Schell (19) on intact cells grown to stationary phase in YTSS.

helper strain *E. coli* HB101(pRK2013) (13) were spread on YTSS agar plates. After growing for 24 h at 30°C, the streptomycin-resistant B1 strain containing pRK404 (designated *Achromobacter* sp. strain B1.1) or rifampicin-resistant B1 strain containing the recombinant plasmid pJH123 (*Achromobacter* sp. strain B1.2) was selected from the triparental mating by growth on VSA plates containing 30 μg of tetracycline per ml and 0.2% sucrose instead of vanillate. Recombinant plasmid pJH123 is composed of pRK404 with a *pglA-phoA* hybrid gene encoding a fusion protein comprising the signal sequence and first 177 residues of the extracellular PglA polygalacturonase of *Pseudomonas solanacearum* fused to residue 14 of mature PhoA (19). As found previously for *E. coli* and *P. solanacearum*, pJH123 directed constitutive synthesis of high levels of alkaline phosphatase activity in *Achromobacter* sp. strain B1 (Table 1).

The growth rates and population levels of strains B1, B1.1, and B1.2 were identical over an 18-day period after inoculation into seawater amended with 0.02% yeast extract (not shown). The retention (stability) of pRK404 and pJH123 in the *Achromobacter* strains was monitored by picking 100 colonies isolated from microcosms on either YTSS or VSA plates and retesting them for growth and blue color on YTSS plates with 30 μg of tetracycline per ml and 30 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside, the chromogenic alkaline phosphatase substrate, per ml.

Short-term phosphorus cycling studies. Water was collected from Sapelo Island or the nearshore of Chub Cay, Berry Islands, Bahamas, in acid-washed Nalgene carboys. At sample collection, water temperatures at Sapelo Island ranged from 19.5 (December 1992) to 28°C (September 1992) and salinity ranged from 17 (November 1992) to 23‰ (April 1993). The Bahamian water temperature was 30°C, and its salinity was 36.5‰ (October 1992). Inorganic phosphorus (PO_4^{3-}) concentrations were measured colorimetrically as described by Murphy and Riley (24). Nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) concentrations were measured with a Technicon AutoAnalyzer II system.

Triplicate flasks containing seawater with natural bacterial populations (i.e., unfiltered seawater) were inoculated with B1.1 or B1.2, while controls received no cells. Bacteria (B1.1 and B1.2) were grown from frozen stocks (−70°C) at 30°C on YTSS plates. Tetracycline was present in plates for B1.1 and B1.2. Colonies were grown in YTSS broth to an A_{600} of 0.5. Cells were harvested by centrifugation, washed twice with half-strength sea salts, and resuspended in a small volume of sterilized (0.2-μm pore size filtered and autoclaved) seawater. Strains were added to the flasks at a final population density equal to 8% of the total population (initial natural bacterial density of 5.0×10^6 cells ml^{-1}). A model organophosphorus compound, glycerol 3-phosphate, was added to flasks at the start of the experiment (1 or 10 μM final concentration). In some experiments, nitrogen was also added as 100 μM ammonium chloride. Flasks were incubated at 25°C and illuminated for 12 h each day from a bank of daylight fluorescent lamps (approximately 500 microeinsteins m^{-2} s^{-1}) or, for the Chub Cay study, in natural sunlight in a flowing-seawater tank on the deck of the R/V *Gyre*. Following 60 h of incubation, a pulse of glycerol 3-phosphate was added to the microcosms at concentrations equivalent to the original addition.

Subsamples were removed for culturable cell counts (1 ml), direct counts (5 ml), inorganic phosphate concentration (10 ml), and phytoplankton biomass determinations (50 to 100 ml) immediately after inoculation and at 12- to 24-h intervals over the next 5 to 8 days. Culturable cell numbers were monitored at each sampling by diluting 1-ml subsamples in half-strength sea salts and plating onto YTSS agar. After incubation at 25°C for 3 days, the number of *Achromobacter* cells was determined by picking 100 colonies from the YTSS plates and evaluating the growth of each on VSA medium containing 300 μg of nalidixic acid per ml supplemented with 75 μg of rifampicin per ml (for strain B1), 500 μg of streptomycin and 30 μg of tetracycline per ml (for strain B1.1), or 75 μg of rifampicin and 30 μg of tetracycline per ml (for strain B1.2). Direct counts were performed on 5-ml subsamples by collecting cells on blackened Nuclepore filters (0.2-μm pore size; 25-mm diameter), staining with acridine orange, and counting cells by epifluorescence microscopy (18). Inorganic phosphate concentrations were measured as described previously (24). Phytoplankton biomass was determined by measuring chlorophyll *a* in particulate matter retained on Whatman GF/F filters with a Turner model 111 fluorometer; values were corrected for phaeopigments (33). For all studies, triplicate flasks of each treatment were assayed at each sampling interval.

Long-term phosphorus cycling studies. Water was collected from Sapelo Island as described above in April 1993. Triplicate flasks containing 1 liter of either whole seawater or seawater filtered through a 1.0-μm-pore-size Nuclepore filter (to remove protozoans and larger organisms) were inoculated with strain B1.1 or B1.2. Seawater was not supplemented with an organophosphorus compound in these studies. Flasks were illuminated for 12 h each day as above. Alkaline phosphatase activity was determined at 1- to 5-day intervals with the fluorogenic alkaline phosphatase substrate methylumbelliferyl phosphate (MUFP; Sigma Chemical Co.). For all assays, 0.5 ml of fresh 1 mM MUFP stock solution and 0.5 ml of 0.1 M Tris buffer (pH 8.0) were added to a 4.0-ml sample of water from the microcosm flasks. Samples were incubated at 25°C for 30 to 90 min, depending on enzyme activity. The potential for net phosphate accumulation in the microcosms was also determined at 1- to 5-day intervals by aseptically removing 10 ml of sample, adding 10 μM glycerol 3-phosphate, incubating in the dark for 12 h (25°C), and assaying for phosphate as described above. Culturable cell numbers and direct counts were monitored at each sampling as described above.

RESULTS

Effect of GEM on phosphate concentrations. A series of experiments were conducted to determine whether or not introduction of *Achromobacter* sp. strain B1.2 (engineered to constitutively express high levels of alkaline phosphatase [Table 1]) into natural bacterial assemblages affected rates of phosphate formation from a model dissolved organic phosphorus compound (DOP), glycerol 3-phosphate. In Sapelo Island water, in situ phosphate concentrations averaged 1.0 μM and ranged as high as 2.8 μM in some samples. In the oligotrophic Bahamian waters, phosphate concentrations averaged 0.03 μM. Since we considered it a possibility that the amplitude of GEM effects on P cycling might, at times, be masked by nitrogen limitation, parallel studies were conducted with supplemental inorganic nitrogen. The ambient N concentration (the sum of NH_4^+, NO_3^-, and NO_2^-) ranged from 2 to 10 μM for the Sapelo Island studies and was 0.5 μM for the Chub Cay study.

Following addition of glycerol 3-phosphate, concentrations of reactive phosphate increased over time in all flask microcosms (Fig. 1). The phosphate concentrations in samples amended with parental strain B1 (Fig. 1A) or plasmid-control strain B1.1 (Fig. 1B to I) were not significantly different from those in samples containing only the natural populations. However, after addition of 10 μM glycerol 3-phosphate, a 1.5- to 2-fold-higher phosphate concentration was observed for up to 36 h of incubation in water samples collected from Sapelo Island and amended with the GEM (strain B1.2) relative to that in samples containing the natural populations alone or the natural populations amended with B1.1 (Fig. 1D and F). A 5- to 10-fold-higher phosphate concentration was observed in seawater samples collected from the Bahamas amended with the GEM than in those containing the natural populations amended with B1.1 or the natural populations alone after 24 h of incubation, respectively (Fig. 1B). The differences in phosphate concentrations were statistically significant (as tested by analysis of variance [ANOVA] at the time of maximum difference; $P < 0.05$ for all experiments). When supplemental N was present, the amplitude of the effect was similar, with an approximately 2-fold-higher phosphate concentration (Fig. 1A, E, and G) up to a 36-fold higher phosphate concentration (Fig. 1C) in microcosms amended with the GEM relative to those with B1 or B1.2 (ANOVA; $P < 0.05$ for all experiments).

When flasks were amended with 1 μM glycerol 3-phosphate, a concentration more typical of ambient DOP in seawater, the phosphate concentrations in microcosms containing the GEM also reached levels significantly higher than for natural populations alone or natural populations amended with B1.1 (Fig. 1H and I) (ANOVA; $P < 0.05$ for both experiments). At this

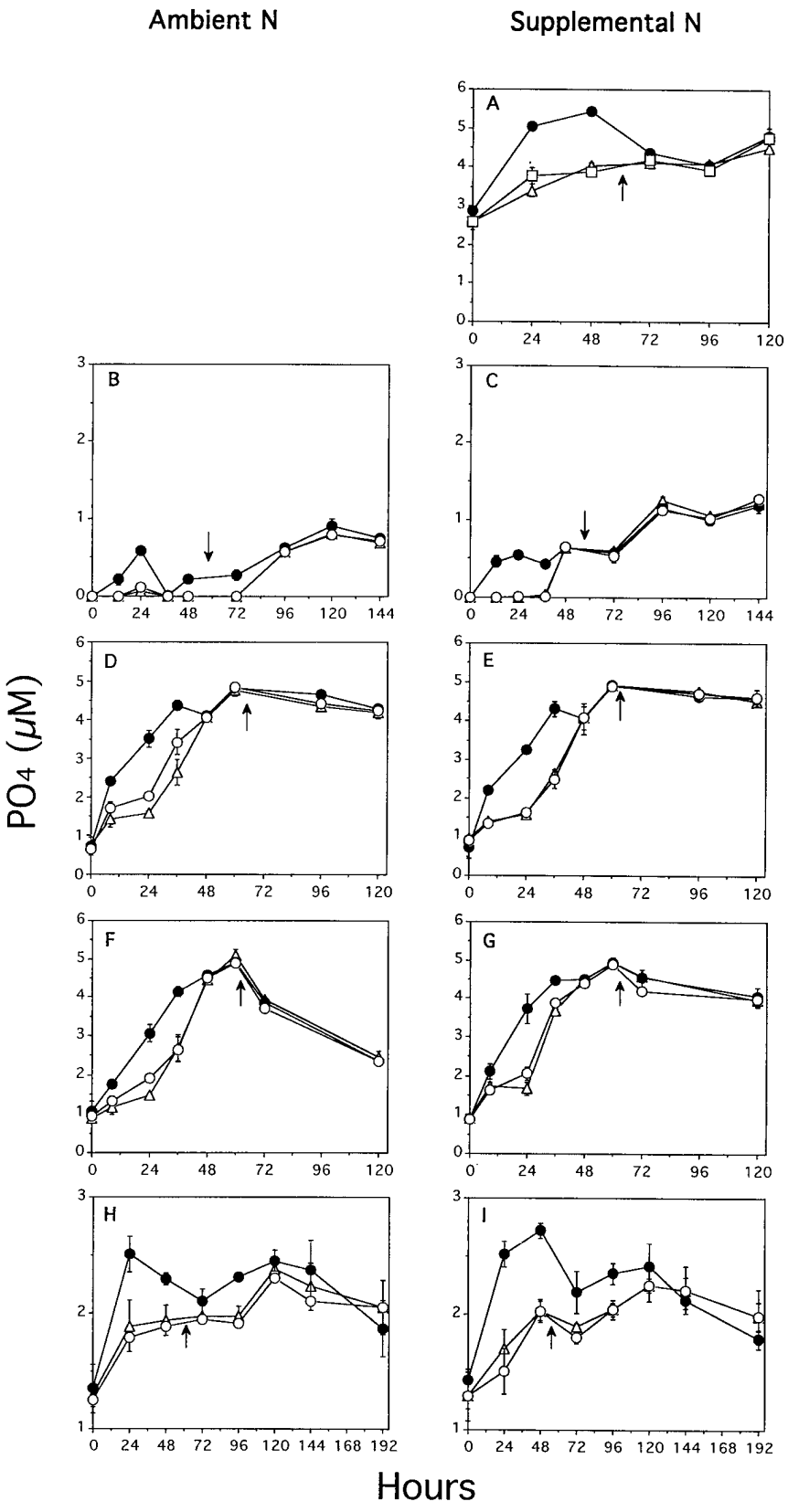

FIG. 1. Accumulation of phosphate in microcosms with natural bacterial populations alone (△) or amended with *Achromobacter* sp. strain B1 (□), B1.1 (○), or B1.2 (●). Experiments were conducted with water collected in September 1992 (A; 10 μM DOP supplement), October 1992 (B and C; 10 μM DOP supplement), November 1992 (D and E; 10 μM DOP supplement), December 1992 (F and G; 10 μM DOP supplement), and April 1993 (H and I; 1 μM DOP supplement). All water was collected from Sapelo Island, Ga., except for the October 1992 collection, which was from Chub Cay, Bahamas. Arrows show time of additional DOP pulse. Vertical bars represent standard errors for three replicate microcosms.

lower concentration of added glycerol 3-phosphate, the presence of the GEM was associated with a 1.5-fold-higher phosphate level both in microcosms with ambient N concentrations and in those with N supplements.

A second pulse of glycerol 3-phosphate (either 10 or 1 μM) added after approximately 60 h resulted in an increase in inorganic phosphate levels for all treatments in the October 1992 and April 1993 studies but no observable change in phosphate concentration in the September 1992, November 1992, and December 1992 studies (Fig. 1). However, only slightly higher concentrations of phosphate were found in seawater flasks containing the GEM than in the other treatments in three of four experiments (Fig. 1B, H, and I). We assume that the bulk of the inorganic phosphate accumulating during these studies was derived from the added glycerol 3-phosphate, although some fraction may have resulted from hydrolysis of naturally occurring DOP. In a parallel series of studies with no glycerol 3-phosphate addition, elevated concentrations of phosphate were not observed either for natural populations alone or for natural populations amended with the GEM (data not shown).

Effect of GEM on phytoplankton biomass. We investigated whether alterations in phosphorus cycling by the engineered *Achromobacter* strains affected primary producers in the microcosms, using the chlorophyll *a* concentration as an index of phytoplankton biomass. After 10 μM glycerol 3-phosphate additions, there were increases of more than fourfold in phytoplankton biomass when the GEM was added to the natural bacterial assemblage relative to additions of plasmid-only control strain B1.1 or to the unamended natural bacterial assemblage (Fig. 2A, B, C, D, E, F, and G) (ANOVA; $P < 0.05$ for all experiments). Phytoplankton biomass was greater when supplemental N was added (Fig. 2B, D, and F) than without N additions (Fig. 2A, C, E, and G), although relative increases in phytoplankton biomass in the presence of the GEM were similar for both treatments (i.e., fourfold). When 1 μM glycerol 3-phosphate was added, an increase in phytoplankton biomass of up to 14-fold occurred in the presence of the GEM relative to the plasmid-only control (B1.1) or the natural assemblage alone (Fig. 2H and I; $P < 0.05$).

Persistence of effects of GEM under nonselective conditions. Nutrient levels in the above experiments deviated from those of natural seawater with regard to elevated DOP and, in some of the studies, N concentrations. We considered it possible that these conditions conveyed a selective advantage for the persistence of the GEM and the activity encoded by its recombinant DNA, which would not be typical of unmanipulated seawater. Therefore, the potential of the GEM for enhancing mineralization of DOP following incubation in microcosms which did not contain supplemental DOP or N over the course of a 35-day study was evaluated.

Alkaline phosphatase activity was greater (by up to 41-fold) in the presence of the GEM than for the natural bacterial population alone, both in whole seawater and in 1.0-μm-filtered seawater (i.e., seawater without large algae, protozoans, and metazoans) (Fig. 3). This difference between treatments persisted for up to 1 month. Enzyme activities in microcosms amended with strain B1.1 were very low and indistinguishable from those for natural populations alone. Likewise, when subsamples from microcosms containing the GEM were amended with DOP and incubated, the resulting phosphate concentrations were up to 3.5-fold (in whole seawater) and 4.5-fold (in 1-μm-filtered seawater) higher than in similarly amended subsamples from microcosms containing strain B1.1 or the natural bacterial populations alone (Fig. 4).

Viable-cell counts made during the study showed that total numbers of culturable bacteria increased transiently but then decreased approximately 10-fold. The pattern for culturable B1.2 cells was similar (Fig. 5). For most of the study, the GEM accounted for a stable 3 to 5% of total culturable cells, both in whole seawater and in 1.0-μm-filtered seawater, perhaps reflecting the natural carrying capacity of the parent strain in coastal seawater. There was no significant difference in culturability between the GEM and the plasmid-only strain in either natural or 1.0-μm-filtered seawater (data not shown).

In both sterilized and natural seawater, the presence of the plasmid alone (strain B1.1) or the plasmid containing the alkaline phosphatase gene (strain B1.2) did not negatively affect population levels in comparison to the wild type (plasmidless) strain B1 (data not shown). Plasmid maintenance in sterile and natural seawater consistently averaged >96% for both *Achromobacter* sp. strains B1.1 and B1.2 (100 colonies tested). Furthermore, of the B1.2 cells which maintained the plasmid, 100% also maintained the *phoA* gene, as evidenced by alkaline phosphatase expression by colonies on YTSS agar (data not shown). Thus, there was no apparent loss or alteration of the recombinant plasmid following introduction of bacterial strains into natural seawater.

DISCUSSION

Previous studies have demonstrated that when certain GEMs derived from laboratory strains (e.g., *E. coli* and *Pseudomonas putida*) are introduced into low-nutrient environments, the numbers of culturable cells decline rapidly, suggesting that they would pose little or no environmental risk (12, 34, 37). Although an introduced GEM might not be able to establish a stable population in the marine environment, it might persist long enough for genetic exchange with members of the indigenous microbial community to occur (1, 11), potentially altering ecosystem structure and function if the foreign DNA were expressed (5, 15, 23). Our study was designed to evaluate the potential for ecosystem-level effects in the event that an indigenous bacterium were to acquire novel DNA from a laboratory-derived GEM or if an indigenous marine strain were altered and reintroduced into its natural habitat.

Our model "indigenous" GEM obtained a plasmid directing constitutive production of alkaline phosphatase via conjugation with *E. coli*. The marine bacterium stably maintained the plasmid when introduced into seawater. Moreover, the presence of a recombinant plasmid that elevated extracytoplasmic alkaline phosphatase production >40-fold relative to that by the parent did not adversely affect the survival of the bacterium (data not shown). Our results are consistent with earlier findings which demonstrated that plasmid maintenance by bacteria in natural waters does not necessarily require artificial selection pressure (8, 21, 32).

Relatively few studies have examined the effects of GEMs on biogeochemical processes in aquatic ecosystems, but modest ecosystem-level effects have been observed upon addition of GEMs to soil. Jones et al. (21) evaluated the impact of GEMs on soil nitrogen cycling and the diversity of microbial populations associated with nitrogen transformations and observed no consistent or significant ecological effects. Addition of a recombinant lignin peroxidase-enhanced *Streptomyces lividans* strain to soil resulted in a transient but detectable increase in the rate of organic carbon mineralization (9, 36). Addition of a *P. putida* engineered to degrade 2,4-dichlorophenoxyacetate to soil microcosms did result in relatively long-term (35 day) changes in total soil CO_2 evolution rates (14).

In the present study, an engineered bacterium sustained a significant change in the rate of a marine biogeochemical pro-

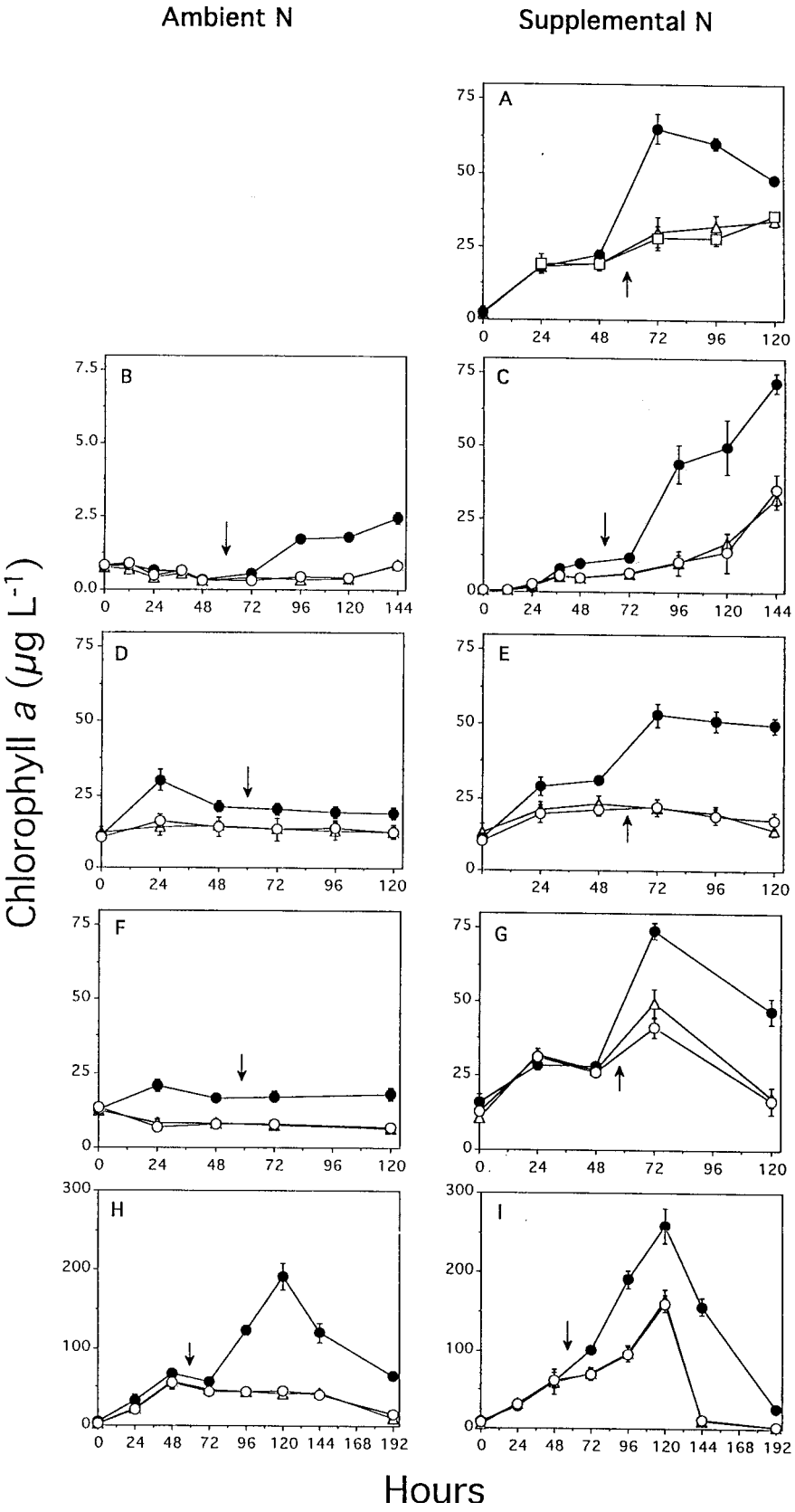

FIG. 2. Changes in phytoplankton biomass in microcosms with natural bacterial populations alone (△) or amended with *Achromobacter* sp. strain B1 (□), B1.1 (○), or B1.2 (●). Sources of water and sampling dates are as described in the legend to Fig. 1. Arrows show time of additional DOP pulse. Vertical bars represent standard errors for three replicate microcosms.

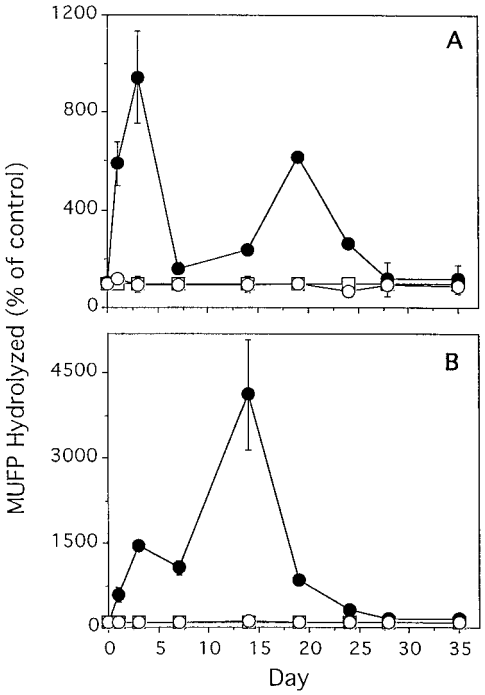

FIG. 3. Alkaline phosphatase activity in subsamples removed from microcosms amended with *Achromobacter* sp. strain B1.1 (○) or B1.2 (●), expressed as a percentage of the activity in subsamples from microcosms with natural bacterial populations alone (□). Studies were conducted in whole seawater (A) and 1.0-μm-filtered seawater (B). Vertical bars represent standard errors for three replicate microcosms.

cess. The direct effect on phosphate availability was reflected in additional changes in the trophic structure of the ecosystem (in this case, in primary producer biomass). It might be argued that the phytoplankton biomass increase was due to indirect effects, such as production of vitamins or other growth factors by the GEM, but this is unlikely since the effect was specific to the presence of *phoA*, and the addition of neither B1 nor B1.1 elevated phytoplankton biomass. The GEM-induced increases

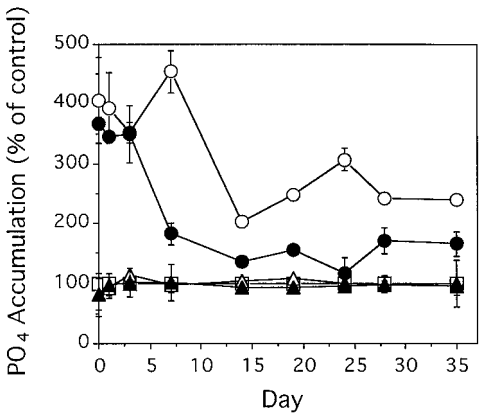

FIG. 4. Phosphate accumulation at the expense of added DOP in subsamples removed from microcosms amended with *Achromobacter* sp. strain B1.1 (△, ▲) or B1.2 (○, ●), expressed as a percentage of the accumulation in subsamples from microcosms with natural bacterial populations alone (□, ■). Solid symbols indicate studies conducted in whole seawater, and open symbols indicate studies in 1.0-μm-filtered seawater. Vertical bars represent standard errors for three replicate microcosms.

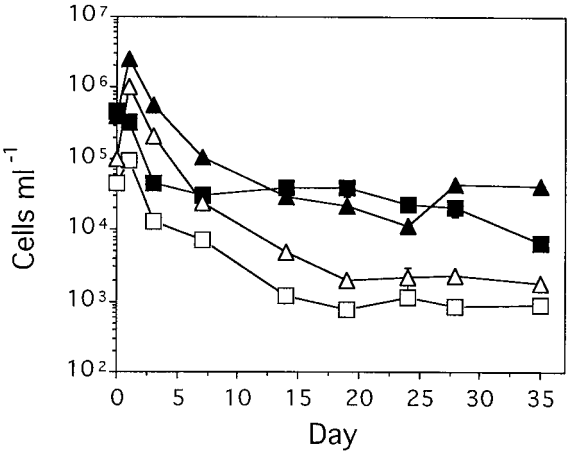

FIG. 5. Culturable cell counts of total bacterial cells (■, ▲) and B1.2 cells (□, △) in whole seawater (squares) and 1-μm-filtered seawater (triangles). Vertical bars represent standard errors for three replicate microcosms.

in phytoplankton biomass were affected by nitrogen availability, and generally nitrogen is considered the limiting nutrient in marine environments (18a), suggesting that the magnitude of the observed ecosystem-level effects of a GEM can be modulated by interactions between different biogeochemical cycles.

The numbers of the GEM culturable from the microcosms decreased 100- to 1,000-fold during the first 3 days before reaching a stable population (Fig. 5), but the potential for phosphate formation decreased by only 2-fold during this time (Fig. 4). Possible explanations for this large discrepancy include an increase in the percentage of viable but nonculturable GEMs after exposure to seawater, an increase in alkaline phosphatase activity per cell, and a transfer of activity from the GEM to indigenous biota. In situ gene transfer between introduced and native bacteria has been demonstrated in several aquatic ecosystems (2, 3, 17, 23, 25, 28), and the fact that the GEM in our study was derived from a common marine organism may have increased the likelihood of transfer.

The simple flask microcosms used in this study included several trophic levels (primary producers, microheterotrophs, and grazers). However, we tracked changes only in phosphate cycling and phytoplankton biomass. It is probable that elevated algal biomass affected the growth of protozoan or metazoan grazers. Thus, our results can be taken as a conservative estimate of the total impact of the GEM on trophic structure in this simplified system; we believe that these results are the first demonstration of sustained ecosystem-level effects of an indigenous GEM in an aquatic ecosystem.

Whether or not the introduction of an engineered genetic sequence into a marine environment will produce significant ecosystem-level changes probably depends on many factors related to the host organism, the specific recombinant DNA carried by the host, and the environmental conditions into which the host is released. In most releases, the GEM might fail to survive, or even if a stable population developed, the recombinant DNA might not be expressed under environmental conditions. Thus, these results cannot be extrapolated to predict potential effects of other GEMs on marine ecosystems. However, they do show that a GEM can induce a measurable, sustained change. In light of the improbability that such releases into open aquatic ecosystems could be effectively contained, a case-by-case assessment of the potential effects of individual GEMs prior to release seems prudent.

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