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Validity of Eucaryote Inhibitors for Assessing Production and Grazing Mortality of Marine Bacterioplankton†

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Application of eucaryote inhibitors to the estimation of production and grazing mortality of bacterioplankton was evaluated. Exposure to a range of concentrations of thiram, cycloheximide, and neutral red (0.4 to 210, 36 to 1,777, 4 to 346 μM, respectively) was 98 to 100% effective at inhibiting growth of a chrysomonad in culture. Exposure to colchicine and griseofulvin (50 to 1,000 μM for both) yielded only 24 to 94 and 53 to 79% inhibition, respectively. Exposures to thiram, neutral red, and griseofulvin were 90 to 100% effective at inhibiting growth in culture of a ciliate, *Cyclidium* sp., and the responses to colchicine and cycloheximide were variable (64 to 100 and 0 to 100% inhibition, respectively). Thiram and neutral red inhibited field populations of nanozooplankton more effectively than cycloheximide and colchicine. Direct effects of eucaryote inhibitors on growing cultures of bacterioplankton varied with parameters measured and duration of exposure. After 3-day exposures, specific growth rates and "instantaneous" heterotrophic potential ([^{14}C]glucose uptake) were not consistently affected, but biosynthetic activity (RNA and DNA syntheses) was depressed. The degree of inhibition of isolates and field populations of phytoplankton depended upon type of inhibitor and phytoplankton species. In field experiments, it was possible to calculate rates of bacterioplankton production and grazing mortality for only 16 of 29 inhibitor experiments and for 4 of 10 size fractionation experiments. Bacterioplankton production and mortality estimates varied greatly with the eucaryote inhibitor used, and those derived from inhibition techniques were substantially different from those derived from fractionation techniques. The poor performances of both techniques are attributed to the following: (i) effects of inhibitors on phytoplankton, (ii) indirect effects of the inhibitors on bacterioplankton, and (iii) insufficient separation of grazers from prey by filtration techniques. Because of the inconsistent results obtained in this investigation, we strongly recommend exercising caution in the application of inhibitor techniques to ecological problems, especially in phototrophically dominated systems.

Selective inhibitors have been used in aquatic microbiology as a method to separate the trophic activity of procaryotes and eucaryotes in planktonic associations (6, 9, 11). Recently, selective eucaryote and procaryote inhibitors have been used to estimate bacterioplankton production rates, to assess the impact of grazing by phagotrophic nano- and microplankton on bacterioplankton production, and to examine bacterial-protistan interactions on sedimenting biogenic particles in marine systems (2, 12, 13, 16; B. F. Sherr, E. B. Sherr, T. L. Andrew, R. D. Fallon, and S. Y. Newell, Mar. Ecol. Prog. Ser., in press).

The use of selective inhibitors has provided a novel approach to the examination of bacterial-protistan interactions. This approach, however, has not been carefully evaluated for marine pelagic systems. Newell et al. (12) incubated coastal surface water samples (collected 0.25 to 15 km from the beaches of Sapelo Island, Ga.) with the eucaryote inhibitors thiram and cycloheximide and compared bacterial numbers in these treatments with uninhibited controls. By their estimates, the proportion of bacterial production grazed by protists was variable, ranging from 0 to 100%. Fuhrman and McManus (2) performed similar experiments, with some refinements, using beach samples from Crane Neck, N.Y., which were incubated with cycloheximide, colchicine, or benzylpenicillin (procaryote inhibitor of cell division). Nongrazing bacterial mortality caused by autolysis and viral or bacterial endoparasites, or both, and grazing mortality were uncoupled from bacterial growth by treatments with benzylpenicillin and eucaryote inhibitors together (nongrazing mortality), treatments with benzylpenicillin alone (nonspecific mortality), and control treatments without inhibitors (growth and mortality). Most of the inferred bacterivory was attributable to small or very flexible organisms (passing through 0.6- and 1.0-μm membranes), and inferred grazing rates for unfiltered samples ranged from 22 to 336% of estimated bacterial production rates ($\bar{x}$ = 123%; standard deviation = 102; growth based on [^{3}H]-thymidine incorporation). Taylor et al. (16) deployed replicate particle interceptor traps at seven depths (50 to 2,000 m) and three stations in the eastern North Pacific. The traps were filled with a nontoxic density gradient solution and half of them were precharged with thiram. Comparisons of concentrations of particulate organic carbon/nitrogen, aplastidic mastigotes, bacteria, and ATP were made among live control traps, live thiram traps, and traps precharged with preservatives. Aplastidic mastigotes were observed to be the numerically dominant phagotrophic organism; comparisons of inhibited and control traps indicated that aplastidic mastigotes accelerated decomposition of sedimenting organic particles and nutrient regeneration.

Theoretically, in the experiments described above, the selective inhibitor debilitates or kills the eucaryotic grazers,

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primarily protists, and thereby uncouples the presumed major agent of bacterial mortality from bacterial production. Inhibitor techniques assume that bacterial growth is not affected by (i) direct chemical inhibition, (ii) use of the inhibitor as a growth substrate, or (iii) indirect community responses such as release of intracellular organic pools by phytoplankton. If any of these assumptions are invalid, the applicability of this technique is seriously compromised.

The present study examines the effects of the eucaryote inhibitors thiram (inhibits protein synthesis), cycloheximide (inhibits 80S ribosomal operation), neutral red (stimulates autocytosis and inhibits endocytosis), colchicine (inhibits microtubule polymerization), and griseofulvin (inhibits microtubule polymerization) on the growth and metabolism of isolated cultures of marine microorganisms. On the basis of laboratory findings, field experiments were conducted with samples collected from meso- and oligotrophic waters at six different geographical sites, using the most reliable inhibitors at their lowest effect doses. For the sake of clarity, the following text utilizes a more precise terminology proposed in reference 14, in which the terms protist, mastigote, plastidic, aplastidic, Apico, and Anano supplant protozoa, flagellate, phototrophic, heterotrophic, Hpico, and Hnano, respectively.

MATERIALS AND METHODS

Culture maintenance. An unidentified aplastidic chrysomonad species (mastigote) and a Scuticociliatida species, *Cyclidium* sp. (ciliate), isolated from oligotrophic waters (~900 miles [1,448 km] northeast of Hawaii), were maintained in a two-stage continuous culture system. In the first stage, a mixed bacterial assemblage obtained from the <0.8-μm filtrate (Nuclepore membrane) of an offshore water sample was grown in f/2 medium (3), which was made from filtered oligotrophic seawater and amended with 3.0 and 1.6 mg of Casamino Acids and sucrose, respectively, liter^{-1}. Outflow from the first stage was split equally to supply two protist culturing vessels (one for *Cyclidium* sp. and one for the chrysomonad). Prior to experiments with protists, a series of culture vessels containing filter-sterilized seawater amended with one rice grain (~20 mg) and 1 μM glucose were inoculated with either log-phase bacteria from the first stage of the chemostat or log-phase protists and attendant bacteria (specific growth rate [μ] = 1 to 3 day^{-1}); these samples were incubated for 24 h at 22°C without light or agitation (= preincubation).

Phytoplankton stock cultures (*Thalassiosira pseudonana* clone 3H, *Synechococcus* sp. clone DC2, *Dunaliella tertolectica*, *Phaeodactylum tricornutum*, *Chaetoceros gracile*, and an unidentified chrysophyte) were maintained in f/2 medium at 22°C under a light bank (67.9 microeinsteins m^{-2} s^{-1}).

Inhibitor preparation. Stock solutions of eucaryote inhibitors were prepared in distilled water in the following concentrations (millimolar): thiram, 2.08; cycloheximide, 17.79; neutral red, 3.46; colchicine, 10.00; and griseofulvin, 10.00; they were stored at −20°C. Prior to use, stock solutions were warmed to 60°C to increase solubility of the least soluble inhibitors (thiram, cycloheximide, and griseofulvin), and the required volumes were filter sterilized through disposable 0.2-μm Acrodisc filters (Gelman Sciences, Inc., Ann Arbor, Mich.). Concentrations of thiram, cycloheximide, and griseofulvin reported below are slightly higher than actual concentrations because these inhibitors have low solubilities in water, and some insoluble material was undoubtedly lost during filtration. In preliminary experiments, all inhibitors were supplied by Sigma Chemical Co., St. Louis, Mo. (as reported in reference 2), but at low concentrations the thiram and cycloheximide from Sigma Chemical Co. were found to be 93 to 100% less effective at inhibiting mastigote and ciliate growth than those supplied by Aldrich Chemical Co., Milwaukee, Wis. Therefore, thiram, cycloheximide, and colchicine used in experiments reported below were supplied by Aldrich Chemical Co.

Inhibitor effects on cultured protists. Following the standard preincubation (outlined above), log-phase cultures of the chrysomonad or *Cyclidium* sp. were transferred to six series of four flasks each (five inhibitors and one control series), a range of four concentrations of inhibitors (1.5 to 2.5 orders of magnitude) was introduced, and the series was incubated for 24 (chrysomonad) or 48 (*Cyclidium* sp.) h. Incubations for all experiments, except phytoplankton growth experiments, were conducted in darkness at 22°C without agitation or aeration, unless otherwise noted. Protistan cell concentrations at the beginning and end of incubations with inhibitors were determined from preserved subsamples (2% [vol/vol] borate-buffered formaldehyde, final concentration). Chrysomonads were enumerated (20 fields per sample) by epifluorescence microscopy at ×400 magnification, using acridine orange-stained preparations (5). *Cyclidium* cells were enumerated by assessing 5 to 10 random transects on a Sedgewick-Rafter counting chamber (1.0-ml capacity).

Inhibitor effects on bacterial growth and metabolism. Short-term effects were examined by inoculating bacterioplankton into filter-sterilized seawater amended with 1.0 μM glucose, 0.005% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.), and 0.1 μCi of [2-^{3}H]adenine (specific activity, 20 Ci mmol^{-1}; New England Nuclear Corp., Boston, Mass.) ml^{-1} and incubating for 1 h on a shaker table. Thiram, cycloheximide, or neutral red was then added at its lowest effective concentration (≥95% inhibition of protistan growth; 8, 178, or 4 μM, respectively), and incubations were continued until the ^3H_2O yield was equal to approximately 30% of total added [^{3}H]adenine activity. Biosynthetic activity ([^{3}H]ATP, [^{3}H]RNA, [^{3}H]DNA, and ^3H_2O production) and total ATP concentrations were measured with material captured on Whatman GF/F filters as described in references 7 and 8. Radioactive samples were suspended in Aquasol-2 (New England Nuclear) and assayed on a Tri-Carb 4640 (Packard Instrument Co., Inc., Rockville, Md.), using a channels ratio quench correction program.

Long-term inhibitor effects on growth and biosynthetic activity of bacterioplankton were measured after a 3-day incubation in two subsequent experiments. In the first experiment, bacterioplankton from the continuous culture system was inoculated into four flasks containing 100 ml of filter-sterilized seawater amended with 1.0 μM glucose and a single sterile grain of rice. After a 4-h incubation, the cultures were amended with inhibitors and incubated on a shaker table at 150 rpm. After 3 days, 0.1 μCi of [^{3}H]adenine ml^{-1} was added, and total ATP, [^{3}H]ATP, [^{3}H]RNA, [^{3}H]DNA, and ^3H_2O production were measured after 2.5 h (7, 8). In the second experiment, a mixture of senescent phytoplankton cultures (*C. gracile*, *T. pseudonana*, and an unidentified coccolithophore) replaced rice to simulate natural nutrient sources more closely. Bacteria were enumerated by epifluorescent microscopy from preserved samples which were stained with acridine orange and captured on 0.2-μm Nuclepore membranes (5). Growth rates were calculated assuming exponential growth and using the formula, μ

$= (\ln C_x - \ln C_0)/t_x$, where μ = divisions per day, C_0 and C_x = bacterial concentrations at beginning and end of incubation, and t_x = length of incubation.

Long-term effects of the inhibitors on the heterotrophic potentials of bacterioplankton were examined by using the same protocol described for the first long-term experiment, except that on day 3 each flask was amended with 2.28 μCi of D-[U-^{14}C]glucose (NEC 042X; specific activity, 346 mCi mmol^{-1}; New England Nuclear). [^{14}C]glucose incorporation at 0 and 4 h was determined by the radioactivity of material from 1-ml subsamples captured on a Whatman GF/F filter (16). Respired $^{14}CO_2$ was evolved by syringe injection of 0.1 N HCl into sealed serum bottles containing 15-ml subsamples and was captured on a suspended filter paper wick soaked with β-phenethylamine (4). Samples were radioassayed as described above.

Effects of inhibitors on phytoplankton. Inocula (0.1 ml) from monocultures of marine phytoplankton (listed above) were introduced into triplicate culture tubes containing 10 ml of f/2 medium with or without inhibitors. Cultures were incubated for 7 days as described above, and then in vivo fluorescences were measured on a Turner Design fluorometer. Fluorescences of inhibited treatments were compared with those of controls to calculate inhibition.

Field experiments. During the VERTEX 5 cruise (4 June to 3 July 1984), 30-liter samples were collected with Niskin bottles from the chlorophyll *a* maximum (100 to 135 m) at three stations (A, B, C) and from 10 m at station X on a west-southwest transect from Point Sur, Calif. (northeastern Pacific transect experiment). Stations were 102 to 1,448 km offshore (see reference 16). Macrozooplankton were eliminated in all field experiments by prescreening the samples through a 200-μm Nitex mesh. The sample was then subjected to a concentration-fractionation protocol designed to enhance the influence of nano- and microplankton grazers (2 to 20 and 20 to 200 μm in diameter, respectively). The microplankton fraction (MICRO) was concentrated from 30 to 1 or 2 liters across a 20-μm Nitex mesh by gravity-driven reverse-flow tangential filtration. The nanoplankton fraction (NANO) was concentrated two- to threefold from the <20-μm filtrate across a 2.0-μm Nuclepore membrane (142 mm in diameter). The <2.0-μm filtrate was not concentrated and represented the picoplankton fraction (PICO). Subsamples (100 ml) from each fraction (MICRO, NANO, and PICO) were incubated in acid-washed polyethylene bottles for 24 h in darkness at in situ temperatures after addition of inhibitors. Subsamples from each fraction were preserved in 2% borate-buffered formaldehyde at 0 and 24 h for subsequent epifluorescent microscopic analysis and growth rate calculations (as described above).

In the Hilo coast experiment, a 24-liter sample was collected at a station 6.7 km off Hilo, Hawaii (19 February 1985), with Niskin bottles from 120 and 125 m (chlorophyll *a* maximum). A 4-liter subsample was passed through a 1.0-μm Nitex (nominal pore size) mesh-covered cylindrical frame by reverse-flow filtration as described above and represented a reduced grazer treatment. Duplicate 1-liter aliquots for each treatment were placed in sterile 1.5-liter Nasco Whirl-Pak bags in an effort to minimize containment effects and were treated as follows: (i) <200 μm (MICRO); (ii) <1.0 μm (PICO); (iii) MICRO, 4 μM thiram; (iv) MICRO, 178 μM cycloheximide; (v) MICRO, 4 μM neutral red; (vi) MICRO, 50 μM colchicine. All incubations were conducted under a simulated in situ light and temperature (23.5°C) regime in an on-deck incubator equipped with neutral density filters and flowthrough cooling water. Enumeration of picoplankton and mastigotes was performed on preserved subsamples from 0- and 24-h time points as described above. Mastigotes (30- to 50-ml subsamples) were collected on Irgalan Black-stained 0.8-μm Nuclepore membranes. Chlorophyll *a* was extracted in 90% acetone from replicate GF/F filters that had collected material from 200-ml subsamples taken at 0 and 24 h. Chlorophyll *a* samples were stored at −20°C and subsequently analyzed on a Turner Design fluorometer (15).

Detailed examination of the effects of thiram on the microplankton community was performed on a 25-liter sample collected from the middle of an oligotrophic embayment (Kaneohe Bay, Oahu, Hawaii, 30 October 1984) at 1-m depth. A 12-liter subsample was fractionated through a 2.0-μm Nuclepore membrane by reverse-flow filtration to minimize predator concentrations. Duplicate 2.3-liter aliquots were placed in acid-washed clear polycarbonate bottles to further reduce containment effects. Experimental treatments were as follows: (i) <200 μm (MICRO), T_0; (ii) MICRO, T_{24}; (iii) MICRO, T_{24}, plus 4 μM thiram; (iv) <2.0 μm (PICO), T_0; (v) PICO, T_{24}; (vi) PICO, T_{24}, plus 4 μM thiram; where T_0 and T_{24} are time zero and 24 h, respectively. Incubation bottles were tethered on a weighted line and submerged at a depth of 1 to 2 m off the pier at the Hawaii Institute of Marine Biology, Coconut Island, for 24 h. In addition to quantification of bacterioplankton (Apico), aplastidic mastigotes (Anano), and chlorophyll *a*, enumerations of plastidic picoplankton (Ppico) and plastidic nanoplankton (Pnano) and measurements of microbial ATP were performed. Enumerations of Ppico and Pnano were performed on autofluorescing cells from 30-ml subsamples captured on 0.2-μm darkened Nuclepore membranes. Microbial ATP from duplicate 350-ml subsamples captured on GF/F filters were extracted and analyzed as described in reference 8.

RESULTS

Laboratory studies. (i) Inhibition of protistan growth. Growth of the chrysomonad was 0 to 2% of the controls in the presence of thiram, neutral red, and cycloheximide over the ranges examined (Fig. 1A). Colchicine and griseofulvin, which both inhibit microtubule polymerization, were less effective; growth of the chrysomonad ranged from 24 to 94% of that of the control (Fig. 1A). Growth of the ciliate *Cyclidium* sp. was 0 to 10% of the control in the presence of thiram, neutral red, and griseofulvin over the ranges examined (Fig. 1B). Growth responses of *Cyclidium* sp. to cycloheximide and colchicine were variable and these compounds were less effective at inhibiting growth (Fig. 1B). Thiram and neutral red were, therefore, most effective at inhibiting growth of both types of protists, and these compounds were effective at very low concentrations (4 μM for both).

Cultures of both the mastigote and the ciliate were observed microscopically to determine whether these protists were viable after incubation with the inhibitors. Chrysomonads were observed swimming up to 3 days after addition of thiram at the lowest concentration (4 μM), and *Cyclidium* sp. remained active in all concentrations of cycloheximide and in all but the highest concentration of thiram (208 μM) up to 6 days after inhibitor addition.

(ii) Effects of inhibitors on bacterial metabolism and growth. Over short-term incubations, production of [^{3}H]RNA and [^{3}H]DNA by bacterioplankton cultures was impeded by the presence of thiram, cycloheximide, and neutral red to 28, 68, and 38% of the control, respectively (Fig. 2). ^3H_2O produc-

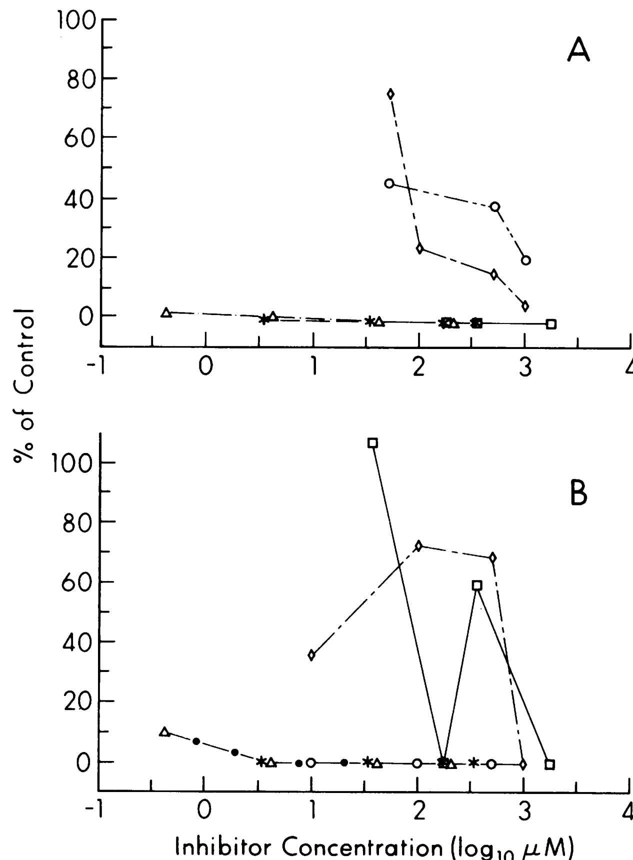

FIG. 1. Dose responses of cultured protistan production to eucaryote inhibitors as percentage of control (mean of 5 to 20 counts). (A) Responses of a phagotrophic chrysomonad to a 24-h exposure; (B) responses of the ciliate *Cyclidium* sp. to a 48-h exposure. Symbols: *, neutral red; △, thiram; □, cycloheximide; ◇, colchicine; ○, griseofulvin.

tion in treatments with inhibitors was similar to control values. On the basis of a close correlation between ^3H_2O production and adenine uptake (8), these results suggest that [^{3}H]adenine was taken up at comparable rates in all treatments but that biosynthesis (as measured by [^{3}H]RNA and [^{3}H]DNA production) was less efficient in the inhibited treatments relative to the control (Fig. 2). Growth rates over the entire incubation were estimated by using changes in ATP and assuming exponential increase. Growth rates ranged from 0.073 to 0.202 h^{-1}, and the inhibitors impeded growth in proportions similar to those of the biosynthesis measurements (Fig. 2).

Since most published inhibitor studies are based on ≥1-day incubations, we were interested in the effects of inhibitors in long-term experiments, such as in particle trap deployments (16). The specific production rates (μ; per day) of ATP and cells for control, thiram, cycloheximide, neutral red, and colchicine treatments after 3 days were similar for bacterioplankton grown on rice detritus (Fig. 3A) and on senescent phytoplankton (Fig. 3B). On the other hand, production rates of [^{3}H]RNA, [^{3}H]DNA, and ^3H_2O in the inhibited treatments were generally lower in the short incubations (<2.5 h) at the end of the 3-day period (Fig. 3A and B). The rates of incorporation and respiration of [^{14}C]glucose at the end of a 3-day incubation period were comparable to control values for the inhibitors thiram, cycloheximide, and neutral red (Fig. 4).

(iii) Effects of inhibitors on phytoplankton. Growth responses of six species of phytoplankton to inhibitors were highly variable among species and between inhibitors (Table 1). The three diatom species were uniformly sensitive to thiram, cycloheximide, and neutral red. Two of these species (*P. tricornutum* and *C. gracile*) were less affected by colchicine; growth rates were 50 and 58% of the control, respectively. The procaryote *Synechococcus* sp. was relatively unaffected by cycloheximide and neutral red but severely inhibited by thiram (μ = 5% of control). The chlorophyte *D. tertolectica* was slightly inhibited by thiram and neutral red and was severely inhibited by cycloheximide (μ = 4% of control). The unidentified chrysophyte was sensitive to thiram (μ = 19% of control) and cycloheximide (μ = 2% of control) and less sensitive to neutral red and colchicine.

Field experiments. (i) Northeastern Pacific transect experiment. For comparative purposes, size fractionation and inhibitor techniques were used concurrently in all field experiments. The PICO fraction in this experiment represented the picoplankton assemblage released from grazing pressure. In the absence of substrate limitation, we expected that positive and similar growth rates would occur in all treatments. Apparent specific growth rates of aplastidic picoplankton (Apico) were positive in 13 of 16 samples (Fig. 5A), and these positive values varied from 0.02 to 1.37 day^{-1}. There was, however, no consistent effect of the inhibitors; i.e., growth in the inhibited treatments varied inconsistently with respect to one another and to the controls (Fig. 5A).

The NANO fraction represented picoplankton in the presence of both nanoplankton predators (Anano; concentrated two to three times) and plastidic nanoplankton (Pnano). We predicted that, if the eucaryote inhibitors were effective, apparent growth rates of Apico would be lower in the controls relative to the inhibited treatments. This was not the case. Apparent growth rates of Apico in the presence of inhibitors exceeded those of controls in only 7 of 12 samples (Fig. 5B). Apparent growth rates were consistently higher than controls only in treatments with the inhibitor cycloheximide. Overall, the apparent growth rates of Apico in this size fraction were higher than in PICO and MICRO fractions, ranging from 0.07 to 1.51 day^{-1}.

The MICRO fraction represented pico- and nanoplankton

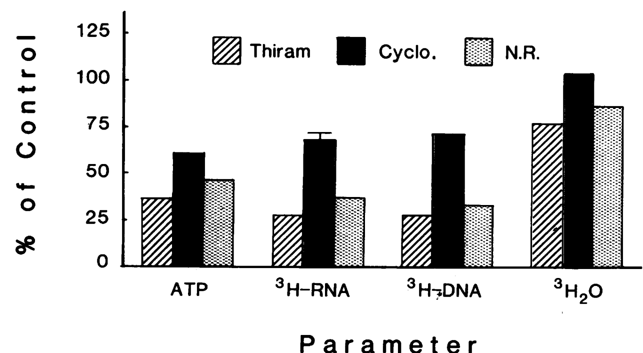

FIG. 2. Short-term (6-h) effects of inhibitors on production of ATP, [^{3}H]RNA, [^{3}H]DNA, and ^3H_2O by laboratory cultures of bacterioplankton: 8 μM thiram; 178 μM cycloheximide (Cyclo.); 4 μM neutral red (N.R.). Bars represent means of two replicates; ranges of most were too narrow to present.

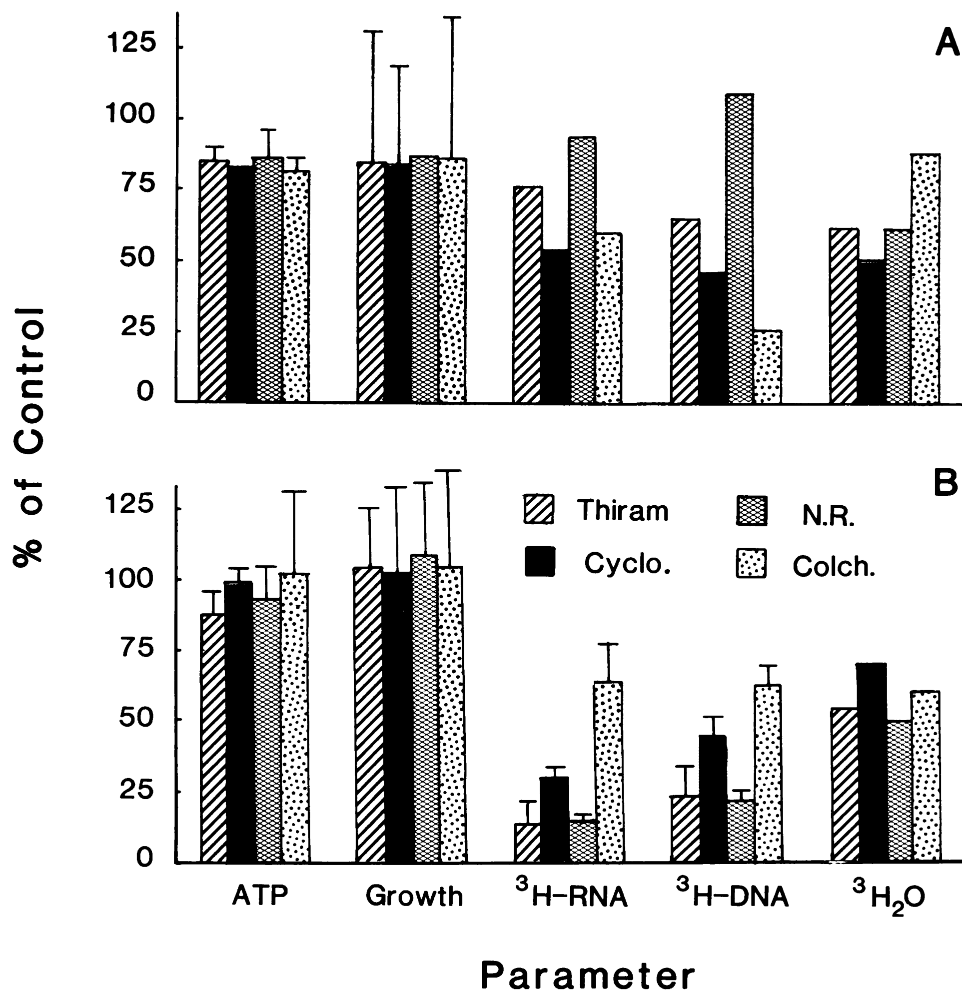

FIG. 3. Long-term (3-day) effects of inhibitors on production rates of ATP, cells, [^{3}H]RNA, [^{3}H]DNA, and ^3H_2O by laboratory cultures of bacterioplankton. Addition of [^{3}H]adenine and radioassay were performed in the last 2.5 (A) or 1 (B) h of incubation. (A) Marine bacterioplankton incubated for 3 days with rice grain, 1 μM glucose, and inhibitors. (B) Same protocol as (A) but senescent phytoplankton used as a nutrient source. Concentrations: 8 μM thiram; 178 μM cycloheximide (Cyclo.); 4 μM neutral red (N.R.); 50 μM colchicine (Colch.). Error bars represent ranges of duplicate samples.

in the presence of enhanced microplankton concentrations (15 to 20 times). Apparent growth rates of Apico were predicted to be higher in the inhibited treatments than in the controls but, as in the NANO size fraction, they were higher in only 7 of 12 inhibited samples (Fig. 5C). Apparent growth rates in the presence of cycloheximide at three stations were again greater than controls, which indicated that either grazing was blocked or bacterial growth was stimulated. The apparent growth rates of Apico in all three size fractions in the presence of the three inhibitors did not match expectations.

TABLE 1. Effects of eucaryote inhibitors on chlorophyll *a* production of phytoplankton isolates[a]

Species	% of control[b]							
	Thiram		Cycloheximide		Neutral Red		Colchicine	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Synechococcus sp. (clone DC2)	5.03	0.22	97.35[c]	15.89	83.77[c]	18.33	NT[d]	NT
D. tertolectica	91.07[c]	102.63	4.21	11.55	81.60[c]	141.89	NT	NT
Unidentified chrysophyte	18.59	3.22	1.73	1.11	94.58[c]	5.77	74.73[c]	31.43
T. pseudonana (clone 3H)	0.17	0.20	0.04	0.04	24.77	16.32	NT	NT
P. tricornutum	1.45	0.61	1.03	0.31	2.65	1.01	50.00	28.28
C. gracile	0.88	1.97	6.38	8.18	1.80	0.15	58.16	68.24

[a] In vivo fluorescence measured after 1 week of incubation of 1% inocula in triplicate culture tubes (100% = 0% inhibition).
[b] 4 μM thiram; 178 μM cycloheximide; 2 μM neutral red; 50 μM colchicine.
[c] Not significantly different from control (Student's t test; $P < 0.05$)
[d] NT, Not tested.

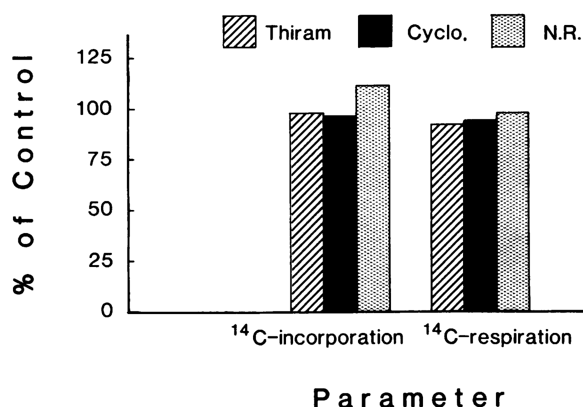

FIG. 4. Long-term effects of inhibitors on potential for incorporation and respiration of [U-^{14}C]glucose by laboratory cultures of mixed assemblages of bacterioplankton. Parallel incubation to Fig. 3A, except samples were incubated last 4 h of day 3 with [U-^{14}C]glucose. Concentrations: 8 μM thiram; 178 μM cycloheximide (Cyclo.); 4 μM neutral red (N.R.). Bars represent means of duplicates; ranges were too narrow to present.

(ii) Hilo coast experiment. At the initiation of the experiment, the PICO (<1.0 m) and MICRO (<200 μm) fractions contained nearly the same Apico (mean ± 95% confidence interval, n = 40 counts; 5.0 ± 2.4 × 10^8 and 4.2 ± 0.9 × 10^8 liter^{-1}) and chlorophyll *a* (0.163 ± 0.110 and 0.142 ± 0.096 μg liter^{-1}) concentrations. The reverse-flow filtration procedure also did not totally eliminate mastigotes from the PICO fraction which contained 2.14 ± 0.71 × 10^5 versus 6.22 ± 1.19 × 10^5 cells liter^{-1} in the MICRO fraction. Hence, grazing pressure on picoplankton was lowered but not eliminated in the PICO fraction. As in the previous field experiment, apparent growth rates of Apico were not enhanced in the PICO fraction or in the MICRO fractions amended with inhibitors relative to the controls (Fig. 6A). Apparent Apico specific growth rates were positive in all treatments, and mean cell volumes increased two- to threefold over the 24-h incubation. The increase in cell volume suggests that containment effects, such as organics leaching from Whirl-Pak bags, may have influenced the results of the experiment.

Growth of mastigotes was positive in the control treatments, and growth was effectively blocked by thiram, cycloheximide, neutral red, and colchicine (Fig. 6B). Apparent specific growth rates of phototrophs (primarily chroococcoid cyanobacteria, *Chlorella*-like chlorophytes, and plastidic mastigotes) as measured by chlorophyll *a* production were negative in all treatments except thiram (Fig. 6C). Chlorophyll *a* concentrations could not be determined in the neutral red treatment because of interference resulting from the stain. The nearly uniform decrease in chlorophyll *a* again suggests that containment effects may have influenced the results of the experiment.

(iii) Kaneohe Bay experiment: community effects of thiram. ATP concentrations increased in all treatments during the 24-h incubation; μ_{ATP} varied from 0.03 to 1.374 day^{-1} (Table 2), indicating net growth of the community or some component thereof. Although apparent growth rates in both PICO treatments were greater than in the MICRO treatments, initial ATP concentrations in the PICO fraction were 22% of the MICRO fraction (82 versus 380 ng liter^{-1}), and final ATP concentrations were 46 to 84% of the MICRO treatments. The net rate of ATP accumulation was not affected by thiram in the PICO fraction but was inhibited in the MICRO fraction (6% of control). At the initiation of the experiment, both fractions contained the same chlorophyll *a* concentrations (0.196 ± 0.029 μg liter^{-1}). Ppico accounted for a substantial portion of the chlorophyll *a* in Kaneohe Bay, and Pnano cells did pass through the 2-μm filter. Initial concentrations of Pnano were 6.96 ± 3.23 × 10^6 and 10.42 ± 5.22 × 10^6 liter^{-1} in the PICO and MICRO fractions, respectively, indicating that only 40% of these organisms were effectively excluded by our reverse-flow fractionation. Specific chlorophyll *a* production was inhibited in the presence of thiram relative to the controls in both size fractions (Table 2).

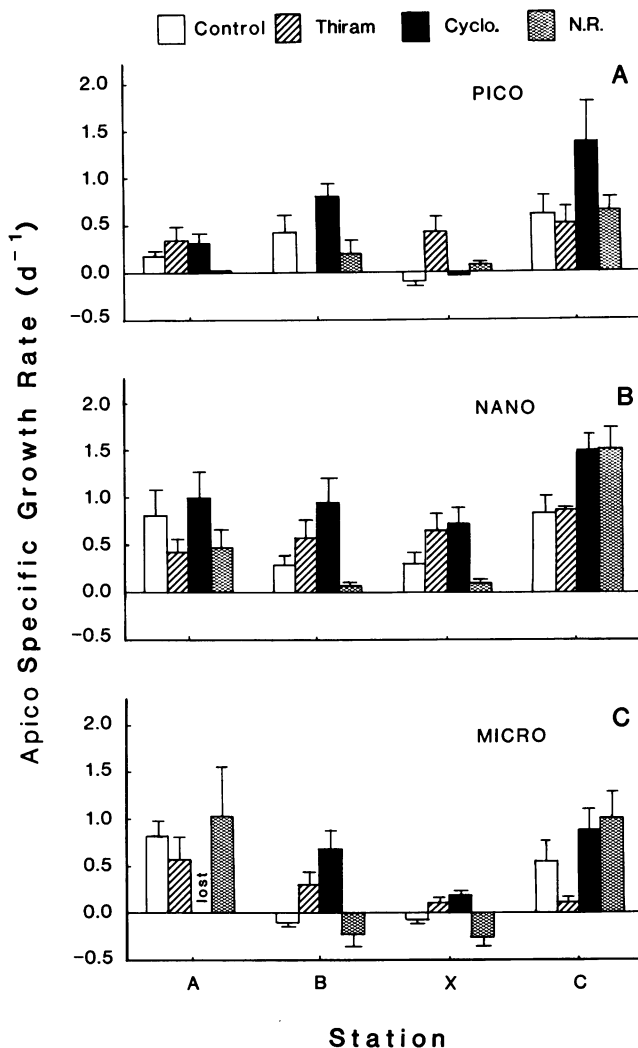

FIG. 5. North Pacific transect experiment. Effects of inhibitors on production of aplastidic picoplankton (Apico) in three size fractions of samples collected from four stations. (A) PICO fraction = 0.2 to 2.0 μm. (B) NANO = 2.0 to 20 μm (concentrated two- to threefold plus PICO fraction). (C) MICRO = 20 to 200 μm (concentrated 15- to 30-fold) plus NANO and PICO fractions (see text for description). Station locations, sample depth, and date: A—35°55'N, 122°34'W, z = 100 m, 16 June 1984; B—34°45'N, 129°54'W, Z = 120 m, 25 June 1984; X—34°11'N, 133°15'W, z = 10 m, 9 June 1984; C—33°06'N, 139°34'W, z = 125 m, 30 June 1984. Samples incubated for 24 h with inhibitors (4, 178, or 2 μM thiram, cycloheximide [Cyclo.], or neutral red [N.R.]) in darkness at ambient temperature. Error bars represent 95% confidence intervals around means (n = 20 fields). d, Day.

TABLE 2. Estimated net production rates for components of the microplanktonic community based on ATP, chlorophyll *a*, and cell concentrations[a]

Parameter[b]	Mean net production rate per day (range)			
	PICO (0.2–2.0 μm)		MICRO (0.2–200 μm)	
	Control	+Thiram	Control	+Thiram
ATP	1.328 (0.073)	1.374 (0.210)	0.538 (0.265)	0.030 (0.033)
Chlorophyll *a*	0.668 (0.491)	−0.807 (0.017)	0.862 (0.030)	0.159 (0.043)
Apico vol[c]	0.545 (0.149)	0.818 (0.025)	0.482 (0.062)	0.771 (0.009)
Ppico	0.102 (0.071)	−0.400 (0.065)	0.086 (0.087)	−0.206 (0.051)
Anano	3.113 (0.046)	−1.972 (0.752)	1.171 (0.146)	−3.190 (0.247)
Pnano	0.523 (0.102)	0.040 (0.083)	0.360 (0.057)	−0.009 (0.000)

[a] Sample was collected from Kaneohe Bay, Oahu, Hawaii (9 November 1984), size fractionated, and incubated for 24 h with or without thiram (4.2 μM). Mean and ranges for two replicate bottles are reported.

[b] Apico, Aplastidic picoplankton (0.2 to 2.0 μm in diameter); Ppico, plastidic picoplankton (autofluorescent, 0.2 to 2.0 μm in diameter); Anano, aplastidic nanoplankton (2 to 20 μm in diameter); Pnano, plastidic nanoplankton (autofluorescent, 2 to 20 μm in diameter).

[c] Apico volume was used in calculation because mean cell volumes varied between treatments for bacteria, but did not for other organisms.

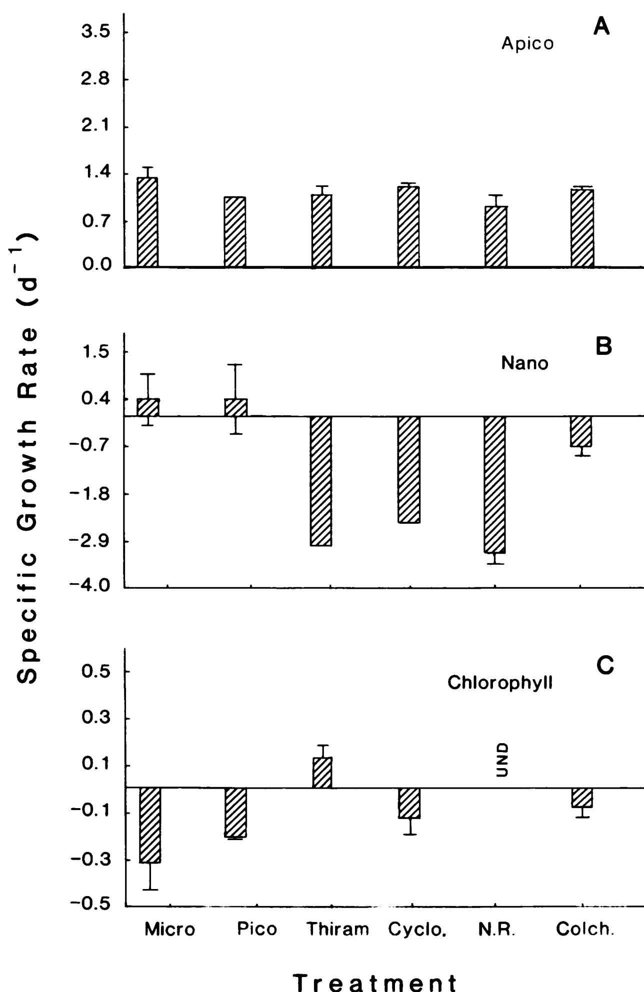

FIG. 6. Hilo Coast experiment. Effects of inhibitors and size fractionation on apparent production rates of Apico (A), nanomastigotes (B), and chlorophyll *a* (C). Sample collected from 120 to 125 m at a station 6.7 km northeast of Hilo, Hawaii, on 16 February 1985. Error bars represent 95% confidence intervals around means (n = 20, 40, and 4 in A, B, and C, respectively). Cyclo., Cycloheximide; N.R., neutral red; Colch., colchicine; UND, Not detectable.

Apico cell concentrations and mean cell volumes in the PICO and MICRO fractions were indistinguishable at the initiation of the experiment (1.20×10^9 and 1.22×10^9 liter^{-1}, respectively, and 0.147 μm^3 cell^{-1} for both). After 24 h, Apico cell concentrations increased uniformly in all treatments and fractions (2.00×10^9 to 2.26×10^9 liter^{-1}). Mean cell volumes, however, in the thiram treatments were 1.4-fold greater than in the controls (0.194 and 0.147 μm^3 cell^{-1}, respectively). On the basis of total biovolume produced, specific growth rates of Apico in thiram treatments of PICO and MICRO fractions were 1.5- to 1.6-fold greater than those of the controls. Net growth rates in the PICO fractions were greater than in comparable MICRO fractions. At the initiation of the experiment, concentrations of Ppico, composed mostly of chroococcoid cyanobacteria and, to a lesser extent, *Chlorella*-like cells, were the same in PICO and MICRO fractions, $9.59 \pm 1.66 \times 10^7$ and $8.58 \pm 1.91 \times 10^7$ liter^{-1}, respectively. Growth rates of Ppico were positive in the control treatments and approximately equal in the PICO and MICRO fractions (Table 2). Apparent growth rates of Ppico in the thiram treatments were negative in both size fractions. A similar negative impact of thiram on the procaryote *Synechococcus* sp. was observed in the laboratory experiments.

Prefractionation excluded 82% of the Anano from the PICO fraction; initial concentrations were $0.61 \pm 0.14 \times 10^5$ and $2.43 \pm 0.30 \times 10^5$ liter^{-1} in the PICO and MICRO fractions, respectively. Unlike Pnano cells, the Anano cells did not tend to pass through the filter. This difference can be attributed to the observed overall larger size of Anano cells relative to Pnano (many Anano cells were in the 2- to 5-μm size range) and possibly to a greater abundance of individuals with rigid cell walls among the Anano. Thiram effectively blocked growth of Anano and Pnano in both fractions (Table 2). Anano were especially sensitive to the inhibitor. It is not clear why there was an apparent difference in the sensitivity of Anano and Pnano. Since thiram inhibits protein synthesis, its effect should be independent of trophic mode. In the control treatments, net growth rates of both Anano and Pnano were high (Table 2). Anano growth rates were especially high in the PICO relative to the MICRO fraction (Table 2), reflecting both the rapid increase in their prey and, probably, release from predation.

In this experiment the effect of thiram on Apico growth appeared to conform with expectation. Apparent growth in the inhibited treatment in both size fractions exceeded growth in the control, and the difference in growth rates might have been

due to grazing. It is also clear, however, that thiram had a negative effect on the phototrophs and that Apico cells in the thiram treatment were larger at the end of the experiment. We attribute this difference in part to stimulation of the Apico cells as a result of dying phytoplankton.

DISCUSSION

On the basis of our laboratory results, the requirements for an inhibitor that blocks protistan activity completely and selectively were not entirely met by any of the inhibitors tested. At the concentrations required to block protistan growth, the inhibitors also affected bacterial metabolism and phytoplankton growth. Sherr et al. (in press) also observed that several eucaryote inhibitors, including thiram, cycloheximide, demicolchicine, and colchicine, had mixed effects on the growth of a ciliate, *Uronema* sp., an aplastidic mastigote, natural assemblages of bacterioplankton, and aplastidic nanoplankton. They found that colchicine and cycloheximide, when used in combination, blocked growth of lab and field populations of protists to varying degrees and did not affect the growth rates of bacterioplankton in the Duplin River estuary. Similarly, Sanders and Porter (13) report that use of cycloheximide and penicillin to assess grazing mortality and production of bacterioplankton in Lake Oglethorpe, Ga., yielded inconsistent results. Furthermore, they demonstrated that three freshwater species of ciliates continued swimming and feeding in the presence of cycloheximide (13). Our own observations demonstrate that, although protistan cell concentrations remained constant or declined during incubations with inhibitors, growth-incompetent protists remained viable for long periods of time. The possibility cannot be excluded that these protists continue to feed, resulting in underestimates of bacterial growth and grazing mortality.

The inhibitors had a strong negative effect on instantaneous measurements of bacterial biosynthesis in both the short- and long-term experiments, whereas substrate utilization and growth rates calculated from ATP and cell yield in the inhibited and control cultures were similar. The effect of inhibitors was, therefore, to alter bacterial growth patterns. Although all cultures eventually reached the same endpoint in 3 days, the rate at which they reached the endpoint may have varied, which violates an important assumption that these inhibitors have no effect on procaryotes. This effect is likely to be most critical in shorter incubations (≤24 h) in which the rate of bacterial growth in inhibited treatments is the key variable for determining grazing and production rates. Also, the inconsistent impact of these inhibitors on phytoplankton isolates suggests that field experiments that use inhibitors to uncouple bacteria-protistan interactions are likely to be influenced indirectly by inhibitor effects on the phytoplankton.

Our field experiments illustrate the interpretational problems associated with inhibitor experiments in planktonic systems. If we assume that the production rates (k) of Apico are approximated by production rates calculated for inhibited treatments and that production rates calculated for the control represent realized production with grazing mortality (r), then we can calculate grazing rates (d) from the difference: $d = k - r$ (equation 1). For the fractionation studies (PICO, NANO, MICRO treatments), the same approach can be applied where k is the production rate in the PICO fraction and r is the realized production rate in the NANO or MICRO fraction. These rates can be derived in each type of experiment by solving the equation: $P_t = P_0 e^{(k - d)t}$ (equation 2), where P is the concentration of the organisms of interest (i.e., Apico) at the beginning (t_0) and end (t_t) of the experiment (10). These rates are calculated for our field experiments for cases in which k was greater than r (Table 3). In the northeastern Pacific transect experiment, k was less than r in 9 of 35 inhibited treatments and in 5 of 8 fractionations. In the experiment off the Hilo coast, k was less than or very nearly equal to r in every case. Calculation of d in these cases would lead to the conclusion that production rates were greater in the presence of grazing mortality than in its absence, which cannot be demonstrated in these experiments. Where calculation of the rates was possible, the values for production and grazing mortality within a single size fraction vary inconsistently among treatments. For example, at station C in the NANO size fraction there is a two-fold difference in the values of k measured with thiram relative to cycloheximide and neutral red and a 20-fold difference in the values of d. The good agreement in the rates

TABLE 3. Estimated Apico production (k) and mortality (d) rates determined by inhibitor and fractionation techniques for field samples

Sample[a]	Treatment[b]	k (day $^{-1}$)	d (day $^{-1}$)	Grazer impact (% of daily production)
N. Pacific transect				
Station A				
PICO	T	0.350	0.156	45
	C	0.313	0.119	38
NANO	C	1.00	0.184	18
MICRO	N	1.03	0.198	19
Station B				
PICO	C	0.796	0.379	48
NANO	T	0.571	0.296	52
	C	0.948	0.673	71
	F	0.417	0.142	34
MICRO	T	0.288	0.388	135
	C	0.677	0.777	115
	F	0.417	0.517	124
Station X				
PICO	T	0.421	0.521	124
	N	0.085	0.185	218
NANO	T	0.647	0.353	55
	C	0.717	0.423	59
MICRO	T	0.096	0.176	183
	C	0.188	0.268	143
Station C				
PICO	C	1.370	0.760	55
	N	0.652	0.042	6
NANO	T	0.874	0.032	4
	C	1.500	0.658	44
	N	1.510	0.668	44
MICRO	C	0.884	0.347	39
	N	1.010	0.473	47
	F	0.610	0.073	12
Hilo Coast		Cannot be calculated		
Kaneohe Bay				
PICO	T	0.818	0.273	33
MICRO	T	0.771	0.289	37
	F	0.545	0.063	12

[a] Prefractionated samples: PICO = 0.2 to 2.0 μm; NANO = 0.2 to 20.0 μm; MICRO = 0.2 to 20 μm.
[b] T, Thiram; C, cycloheximide; N, neutral red; F, fractionation (apparent growth from PICO fraction used as k).

calculated for the inhibitors neutral red and cycloheximide in this case is the exception rather than the rule (Table 3). In the Kaneohe Bay experiment, there is a rough agreement between the production rates (k) calculated with the fractionation procedure and the inhibition method (Table 3, T and F treatments). Grazing mortality (d) calculated by the two methods differs by a factor of 5. The inconsistency of these rates argues that these estimates are largely spurious and that the techniques are not reliable.

It is possible to criticize our field experiments and suggest that the disparities observed were the result of artifacts. For example, in the northeastern Pacific transect experiment, two potential artifacts may have biased our results. First, the 24-h incubations were carried out in 100-ml polyethylene bottles, which may have resulted in containment effects (1). In addition, our reverse-flow fractionation may have increased nutrient concentrations by disrupting cells or by leaching primary amines from Nuclepore membranes (1). In the latter cases, the final fraction (PICO) should have contained the highest concentrations of extracellular metabolites and primary amines and thereby exhibited the highest growth rates. We consistently observed lower Apico growth rates in the PICO fraction and believe that any disruption of cells or leaching resulting from our procedure was minimal. In the other two field experiments, we attempted to minimize containment effects by using sterile 1.5-liter Whirl-Pak bags and acid-washed 2.5-liter polycarbonate bottles, and we still obtained inconsistent results.

Despite possible experimental artifacts, we believe the inhibitor method failed largely because of effects on the phytoplankton, which confounded the experiments. This supposition is supported by the observed sensitivity of laboratory cultures of phytoplankton to inhibitors and the repeated observations of net mortality of phytoplankton and stimulation of bacteria in terms of production and increased average size in the presence of inhibitors. Findings reported in reference 11 also support this hypothesis. They report a variable response of picoplankton from the Sargasso Sea and the Canadian Arctic to a 6-h exposure to cycloheximide (100 μM); ^{3}H-labeled amino acid and [^{3}H]glucose incorporations were only inhibited slightly, whereas $^{33}PO_4$ incorporation was markedly depressed. Some inhibition of $^{33}PO_4$ incorporation may be attributable to altered Ppico activity, but it seems unlikely because incorporation of [^{14}C]bicarbonate by picoplankton was unaffected by cycloheximide. Incorporation of [^{14}C]bicarbonate into Pmicro, however, was totally inhibited by cycloheximide. These observations are consistent with our findings that the cyanobacterium *Synechococcus* sp. (a numerically significant component of Ppico) was not inhibited by cycloheximide, whereas all eucaryotic phytoplankton species tested (some of which may have been included in Li and Dickie's microplankton fraction [11]) were sensitive to cycloheximide.

Use of an additional procaryote inhibitor, such as benzylpenicillin (2, 13; Sherr et al., in press), to block bacterial cell division may circumvent the problem of stimulated bacterial growth but is more likely to introduce a new set of artifacts. Penicillin has been shown to inhibit ingestion of latex beads by three freshwater species of ciliates relative to controls (13). Furthermore, Iturriaga and Zsolnay (6) demonstrated that the procaryote inhibitor gentamicin, which inhibits bacterial metabolism, also inhibits [^{14}C]-bicarbonate incorporation by phytoplankton at concentrations of >214 μM. The problems associated with phytoplankton inhibition potentially compromise results from epipelagic systems but are probably avoided in systems in which phototrophic processes do not dominate, such as below the photic zone, in sediments, and in turbid detritus-based estuaries (16; Sherr et al., in press).

Conclusions. We conclude that the application of inhibitor techniques to epipelagic systems does not produce reliable results and that careful use of these techniques should probably be restricted to situations in which phototrophic processes are not important, such as subeuphotic zone samples and sediments. Even with these restrictions, rate measurements must be interpreted cautiously, because of the presence of nontarget organisms and because of the differential effects of inhibitors on target organisms.

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