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SUMMARY

Investigations were conducted into the effect of 3 marine bacteria, Vibrio anguillarum *#19264,* V. anguillarum *#19109, and* Escherichia coli, *on the growth of 10 phytoplankters. A disc method on agar plates was used to evaluate growth responses. Growth enhancement of all algae in the presence of* V. anguillarum *#19264 occurred on an enriched agar medium; the other bacteria yielded variable responses. Evidence available is consistent with the hypothesis that growth enhancement of algae is related to the release of stimulating substances through bacterial hydrolysis of the agar.*

INTRODUCTION

The introduction of pure culture techniques into algal cultivation in the late 19th century by Beijerinck and the subsequent development and utilization of these techniques by Pringsheim (***36,37***) and numerous other investigators resulted in a wealth of information on nutritional requirements of many species of algae in laboratory culture. Although some nutritional studies have been conducted with bacterized cultures (***1,17,23***), the most widely held opinion is that nutritional, biochemical, and physiological studies should be conducted with axenic strains so that results do not become obscured by a contaminating population. Taxonomic and morphological studies may also be affected by bacterial contaminants. Certain marine bacteria influence the morphological development of *Ulva lactuca* in culture to resemble that of *Enteromorpha* and some bacteria restore normal morphology of *Polysiphonia urceolata* after it has been lost in laboratory culture (***39***). It was also observed that bacteria can induce formation of long filaments and increase development of reproductive structures in *Oedogonium cardiacum* (***27***).

Some investigators question the applicability of data obtained under axenic conditions to conditions in nature because of the complex interactions that occur between organisms in their microenvironments. These cannot be duplicated in the laboratory, but can be isolated from each other and studied independently. Bacteria-algae interactions are particularly important in areas that are organically polluted and apt to contain large numbers of bacteria. Evidence has been accumulating that marine organisms release many substances that can be expected to influence neighboring populations (***25,33,50***). Filtrates from algal cultures may contain organic compounds that could constitute a source of carbon for organisms with heterotrophic capabilities (***21,28,46***), could be a potential nutrient for bacteria (***5,7***), and also regulate bacteria populations through antibiotic production (***9,12,14,45***). There is less evidence of algal productivity that is dependent on bacterial growth, but some authors have attributed enhanced growth of bacterized cultures of marine algae to release of organic growth-promoting factors by bacteria (***7,38***). Waksman *et al.* (***48,49***) noted a frequent correlation of diatom and bacterial populations in pelagic environments and postulated a mutual dependency of these microorganisms. The absence of growth of bacteria-free *Skeletonema costatum* in seawater enriched only with inorganic nutrients was attributed to lack of growth products from normal bacterial populations (***22***). Berland *et al.* (***8***) reported that some marine bacteria also release substances that inhibit algal growth and cause eventual death, *eg*, *Pseudomonas aeruginosa* was toxic for *Tetraselmis striata*. Blasco (***10***) also noted pathogenicity of bacterial contaminants to *Chlorella* in culture.

It is obvious that the result of bacteria-algae interactions must be a species-specific one. This investigation was conducted to determine the effects of bacteria, 2 known pathogens (of fish) and 1 nonpathogen, on the growth of certain phytoplankters in pure culture.

MATERIALS AND METHODS

Axenic cultures of the following species of algae from the Milford culture collection were used in this study: *Nannochloris* sp., *Dunaliella euchlora, Pyramimonas* sp., *Tetraselmis maculata, Stichococcus bacillaris, Phaeodactylum tricornutum, Platymonas* sp., *Chlorella autotrophica, Nitzschia laevis,* and an unknown nonmotile chlorophyte, clone *#U-710*. The bacteria *Vibrio anguillarum* strain #19264 and *V. anguillarum* strain #19109 were obtained from the American Type Culture Collection, Rockville, Maryland. The marine strain of *Escherichia coli* #118-3 was supplied through the courtesy of Dr. Paul Burkholder.

Stock cultures of the algae were maintained under constant cool-white fluorescent lamps (about 500 ft-c) at 18–20 C in the

[1] Trade names referred to in this publication do not imply endorsement of commercial products by the National Marine Fisheries Service.

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following growth medium (designated as E): KH_2PO_4, 20 mg; $NaNO_3$, 310 mg; $Na_2FeEDTA$, 10 mg; tris(hydroxymethyl)-aminomethane, 1 g; thiamine HCl, 0.3 mg; vitamin B_{12}, 3 μg; seawater, 500 ml; distilled water, 500 ml; and a trace metal mix (*19*). Stock cultures of bacteria were incubated at room temperature in the dark, in the following growth medium: Difco[2] purified agar, 15 g; trypticase, 2 g; Bacto-Soytone, 2 g; yeast extract, 2 g; cobalamine, 1 mg; marine mud extract, 100 ml (prepared by autoclaving 1 kg of wet marine mud with 1 liter seawater for 20 min and filtering); seawater, 900 ml. All media were tubed in 19 × 150 mm screw-capped test tubes and autoclaved at 17 lb for 20 min. Tubes of agar media for bacteria were slanted, cooled, and streaked with inocula. Stock cultures of bacteria were subcultured every 1–2 weeks and algae at about 4 weeks. All glassware was Pyrex with the exception of Petri dishes which were presterilized polystyrene.

Growth of algae in response to bacteria was assayed by a disc method. Algal growth medium (E) with the addition of 1.5% Bacto-Agar was sterilized, poured into Petri dishes to a depth of about 3 mm, and allowed to solidify. Tubes containing 10 ml of this agar medium were melted, cooled to about 40 C, and inoculated with 1 ml of a 3- to 4-week algal culture from E medium. These tubes were immediately rotated vigorously, poured over the previously hardened base in the Petri dish, and allowed to solidify at room temperature.

Inocula of the 3 strains of bacteria used for experiments were standardized by the following procedure: bacteria were subcultured from agar stock cultures into 20 ml of broth media (identical to the stock culture media but with agar omitted) in 25-ml Pyrex centrifuge tubes and incubated at room temperature for 24 hr. These cultures were then centrifuged for 30 min at low speeds (3500 *g*) at 5 C. About 15 ml of the supernatant from each tube was decanted aseptically and sedimented cells were pooled to achieve a large enough working volume. Optical densities of samples in sterile, calibrated test tubes were read in a Bausch and Lomb Spectronic 20 at 520 nm. Samples were adjusted to give a standard density by diluting with the decanted sterile supernatant. Aliquots of this suspension were used in making dilution plate counts. Suspensions used to charge experimental discs had average concentrations of: *V. anguillarum* #19109, 4.7×10^8/ml; *V. anguillarum* #19264, 6.6×10^9, *E. coli*, 3.5×10^9.

Paper discs were prepared from Millipore filter support paper (cat. #AP100-47SO, Millipore Corp., Bedford, Mass.) with a standard paper punch, placed in an empty Petri dish and autoclaved for 20 min at 17 lb. Experimental discs were charged aseptically with 25 lambda of the standardized bacteria suspension. To prepare a dry, inactive sample of bacteria for testing one-half of the charged discs were placed in sterile Petri dishes, put into a desiccator over $CaCl_2$ under vacuum for 24 hr at room temperature, or refrigerated at 0 C. Three sets of control discs were prepared: (1) sterile paper discs without any additives, (2) discs charged with 25 lambda of sterile bacteria broth medium, and (3) discs charged with 25 lambda of heat-killed bacteria from the standardized suspension. Killed *V. anguillarum* #19109 and #19264 were prepared by heating suspensions in a water bath at 60 C for 15 min, but for *E. coli* heating at 70 C for 15 min was necessary to kill organisms. Effectiveness of the heat treatment in killing bacteria was determined by inoculating the heat-treated sample into bacterial growth media. Sterile forceps were used to transfer discs to algae-seeded agar plates. Plates were prepared in duplicate and incubated at 18–20 C with constant illumination under cool-white fluorescent lights (about 500 ft-c) for several weeks. At 2, 3, 4, and 8 weeks, growth of algae on plates was observed and zones of inhibition or enhancement of algal growth surrounding each disc on the surface of the plate and directly underneath the disc were noted. Plates were sealed with tape at 3 weeks to prevent evaporation. No change in the basic growth response with time was observed from the earliest to the final observation except for a small increase in density of algal growth.

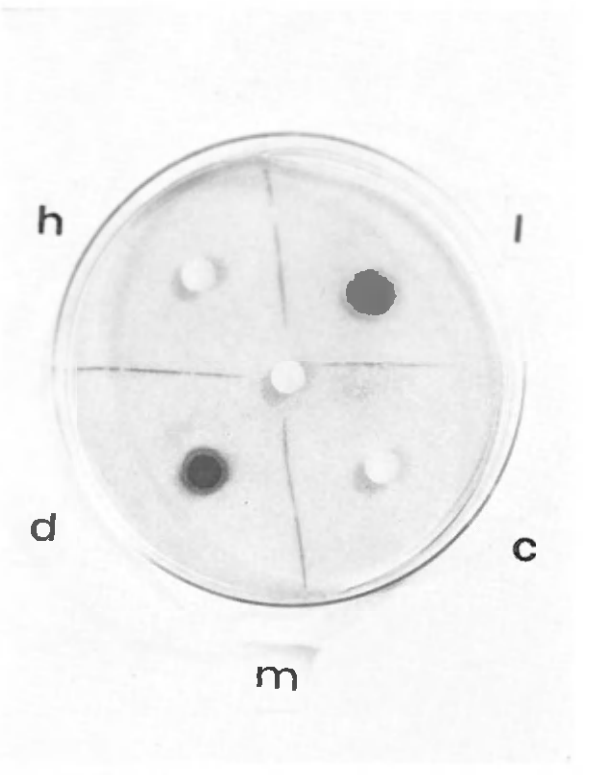

FIG. 1. Surface view of agar plate seeded with *Pyramimonas* sp. and discs charged as follows: *l*, living *E. coli*; *d*, vacuum-dried *E. coli*; *h*, heat-killed *E. coli*; *m*, bacteria growth media; *c*, uncharged control disc.

RESULTS

Growth of all species of algae in agar pour plates was uniform with the exception of *S. bacillaris*, which tended to grow in streaks (although this did not interfere with evaluation of results). Wherever enhancement of algal growth occurred it appeared as a significant zone of dense growth around the periphery of the disc, in some species on the disc itself and partially into the depth of the agar beneath the disc (Fig. 1). There was never any evidence of algal growth stimulation surrounding an untreated disc or a disc charged with sterile bacterial growth medium. Algal responses to the presence of discs charged with bacteria and control discs are summarized in Table 1. Examples of plates photographed through the bottom of algae-seeded plates with discs on the surface are shown in Fig. 2. *V. anguillarum* strain #19264 stimulated growth of every alga tested. Reaction to *V. anguillarum* strain #19109 and to *E. coli* was, in general, less intense and less frequent than with *V. anguillarum* #19264.

Growth enhancement from discs containing vacuum-dried bacteria was often diminished or completely lost. This occurred more frequently if the desiccator was stored overnight at room temperature than if it was stored in the freezer at 0 C. It is noteworthy, however, that *V. anguillarum* #19109 samples had no effect on algal growth in both drying methods.

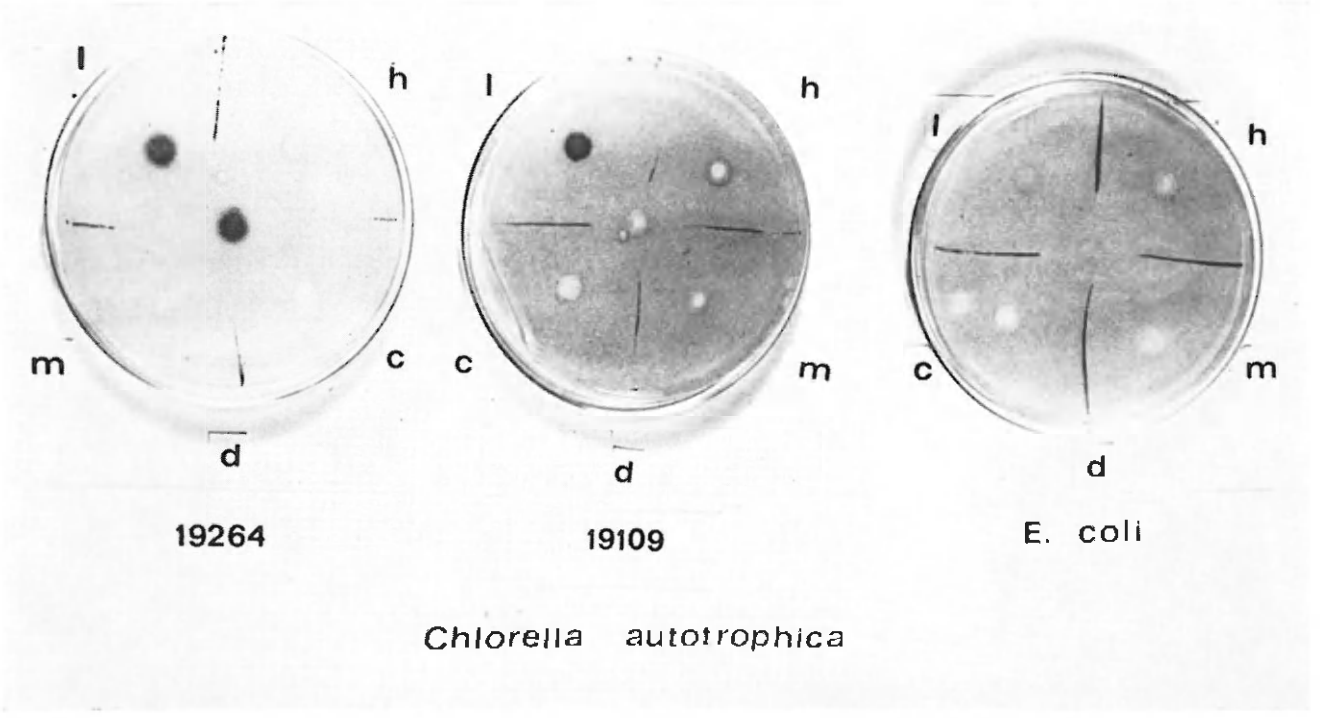

FIG. 2. Bottom view of algae-seeded agar plates with discs on surface. *l*, living bacteria; *d*, vacuum-dried bacteria; *h*, heat-killed bacteria; *m*, bacteria growth medium; *c*, uncharged control disc.

Heating suspensions of bacteria inactivated their algal-stimulating properties in proportion to the length of time the bacteria were exposed to lethal temperatures (Fig. 3).

Growth medium with metabolic products from bacteria cultures was prepared by filtering cultures through a 0.22-μm Millipore filter; the filtrate was collected aseptically and stored at 0 C until needed. Before use the frozen sample was brought to room temperature, 25 lambda charged onto paper discs, and then applied to algae-seeded agar plates. Filtrates from each bacteria culture were tested on every alga, but in no case was growth stimulation evident. All algae tested, with the exception of *P. tricornutum* and *Nitzschia* sp., are chlorophytes and auxotrophy is not prevalent in this group. Nevertheless, experiments were conducted with some algae to determine if there was a possibility that these particular strains were being stimulated through vitamin production by bacteria. Discs charged aseptically with each of the following vitamin solutions in several concentrations, B_{12} (1.0–0.001 μg), thiamine (0.1–0.0001 mg), and biotin (0.005–0.001 mg) were placed on seeded plates of *P. tricornutum*, *C. autotrophica*, *Platymonas* sp., and *T. maculata*. No growth stimulation of algae was observed with these vitamins in the tested concentrations.

Observations all suggested that stimulation of algal growth must be associated with the viability of the bacterial suspension on the disc. Counts of bacteria

TABLE 1. *Radial zones of growth enhancement of algae surrounding discs after 3 weeks.*

Algae	*V. anguillarum* #19264		*V. anguillarum* #19109		*E. coli*		Control	
	Live (mm)	Dried (mm)	Live (mm)	Dried (mm)	Live (mm)	Dried (mm)	Media	Blank
Chlorella autotrophica	2	2	1	0[a]	0	0[a]	0	0
Chlorophyte clone U-710	1.5	0[a]	1	0[a]	0	0[a]	0	0
Nannochloris sp.	1	1	1	0	0.5	0.5	0	0
Stichococcus bacillaris	1	1	1	0	0.5	0.5	0	0
Tetraselmis maculata	2	1	0.5	0	0	0	0	0
Platymonas sp.	1	1	1	0	0.5	0.5	0	0
Pyramimonas sp.	4	4	0	0	2	2	0	0
Dunaliella euchlora	2	2	0.5	0.5	2	2	0	0
Nitzschia sp.	0.5	0[a]	0	0[a]	0.5	0.6[a]	0	0
Phaeodactylum tricornutum	2	2	2	0	2	2	0	0

All discs containing bacteria heated at 60 C or 70 C did not induce zones of enhancement in any of the tested algae.
[a] These discs were dried overnight at room temperature as contrasted with other samples which were dried at 0 C overnight.

TABLE 2. *Viable bacteria on discs after various incubation times.*

Bacteria	Time				
	0 hr	48 hr	1 wk	2 wk	3 wk
E. coli	0.36×10^5	15.9×10^5	19.0×10^5	2.5×10^5	3.4×10^5
V. anguillarum #19109	0.20×10^5	2.7×10^5	4.9×10^5	3.3×10^5	1.3×10^5
V. anguillarum #19264	2.20×10^5	29.7×10^5	10.1×10^5	2.0×10^5	0.59×10^5

made at various times after the discs were charged with the live suspension indeed showed that bacteria remained viable and, in fact, increased in numbers during the first 48 hr of incubation (Table 2).

Some biochemical characteristics of each strain of bacteria are shown in Table 3. The results indicated that, although cellulose was not attacked, cellobiose was fermented. It therefore seemed important to determine if some hydrolysis or other reaction of the bacteria with the paper disc itself was responsible for the release of algal growth-promoting substances. Experiments were conducted in which application of paper discs to the seeded agar surface was eliminated by substituting a procedure in which circular areas of 2 mm diameter × 1 mm deep were cut out of the agar with a sterile glass tube. Bacteria suspensions of 25 lambda were applied directly to the cut out depression in the agar. Evidence of growth stimulation similar to that with the paper disc appeared (Fig. 4).

To obtain information on the time course of algal growth enhancement experiments were conducted in which algae and bacteria were simultaneously inoculated into tubes of artificial seawater medium ASP_2 (*40*). Cultures of *V. anguillarum* #19264 were

TABLE 3. *Biochemical reactions of bacteria.*

	V. anguillarum #19264	*V. anguillarum* #19109	*E. coli* #113-3
Arabinose	+	+	+G
Cellobiose	+	+	+G
Fructose	+	+	+G
Galactose	+	+	+G
Glucose	+	+	+G
Glycerol	+	+	+G
Lactose	+	0	+G
Maltose	+	+	+G
Mannitol	+	+	+G
Mannitose	+	+	+G
Raffinose	+	0	+G
Rhamnose	0	0	+G
Sucrose	+	+	+G
Indole	+	+	+
Methyl-Red Reaction	+	+	+
Urease produced	0	0	0
Acetoin produced	0	0	0
Catalase produced	+	0	+
Cellulose attacked	0	0	0

+ = positive reaction or acid production from sugar; G = gas production from sugar; 0 = negative reaction or no acid production from sugar.

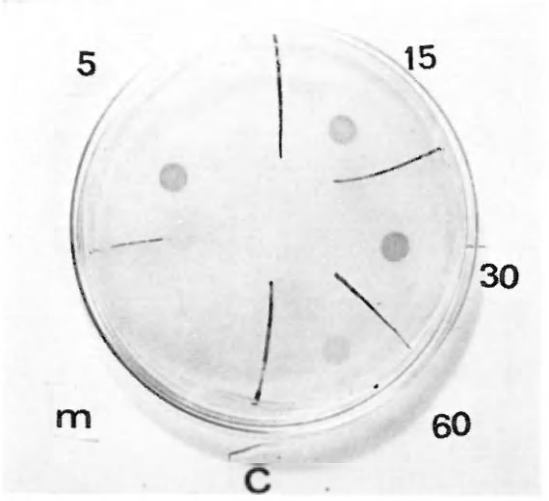

FIG. 3. Response of *D. euchlora* growth to *E. coli* heated at 60 C for 5, 15, 30, and 60 min.

centrifuged, the sediment washed in artificial seawater medium, and inoculated together with the alga. Final concentrations of bacteria were 1.6×10^8 ml; of the alga *C. autotrophica*, 3.3×10^5 ml; *Nannochloris* sp., 4.8×10^5 ml; *P. tricornutum*, 1.3×10^5 ml. Surprisingly, no evidence could be found from plotting daily growth densities of a stimulation of growth rate or of the increase in maximum population of algae with this addition of washed bacterial cells to the algal culture. Some examples of these data are shown in Fig. 5. These results clearly indi-

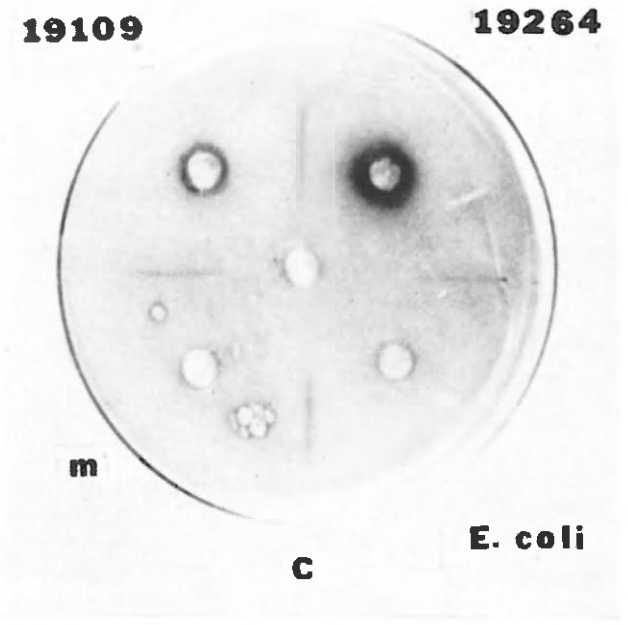

FIG. 4. Growth response of *Chlorella autotrophica* to bacteria in the absence of paper discs, view through bottom of plate; *V. anguillarum* #19264; *V. anguillarum* #19109; *E. coli*; *m*, medium; *c*, blank control.

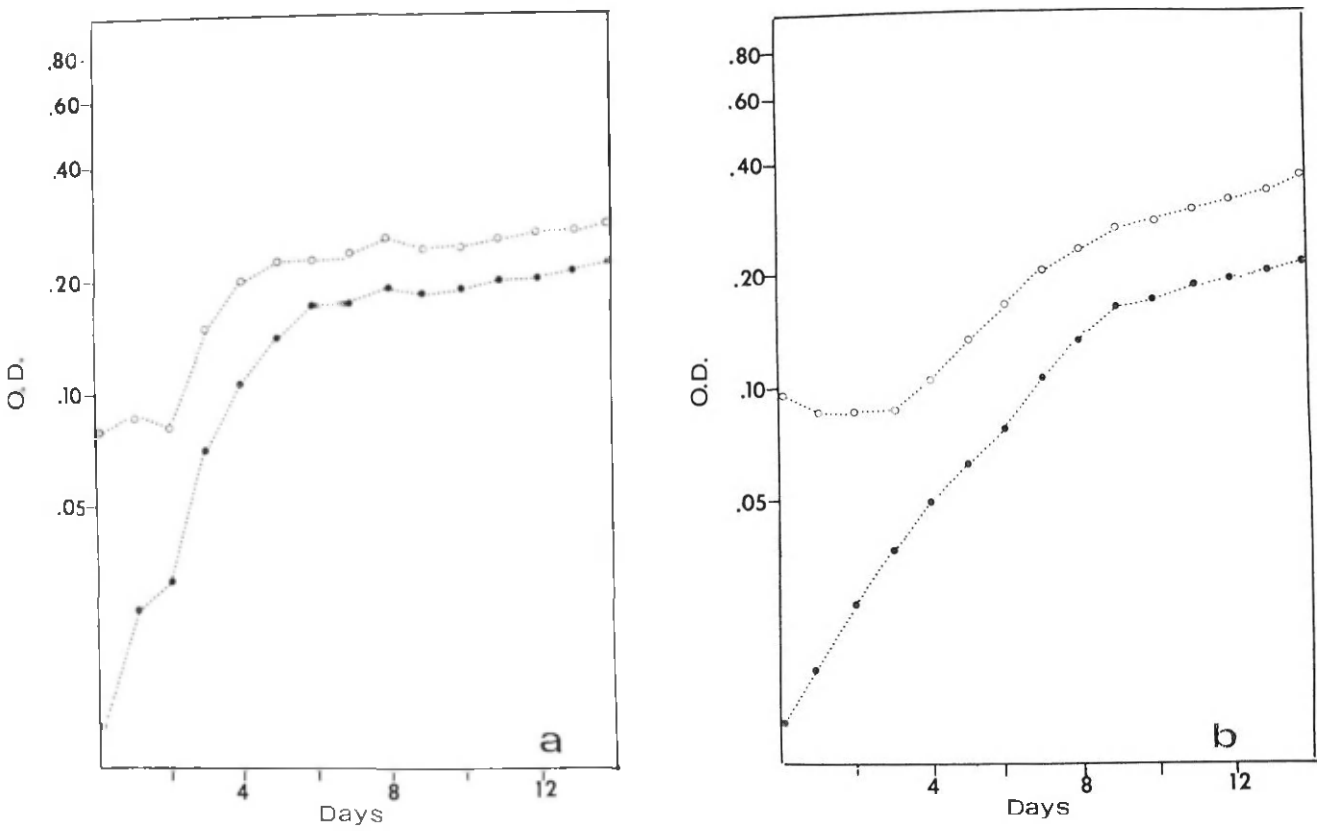

FIG. 5. Growth curves of (*a*) *Nannochloris* sp. and (*b*) *Chlorella autotrophica* with (open circles) and without (closed circles) the addition of a suspension of *V. anguillarum* #19264.

cate that growth stimulation must be a result of material being released through a reaction taking place between a viable bacteria population and its agar growth medium.

FIG. 6. Zones of agar digestion by *V. anguillarum* #19264, *V. anguillarum* #19109, and *E. coli*.

The agar-digesting capabilities of the 3 species of bacteria were evaluated with an iodine test (*18*). Agar plates containing 3 discs on the surface, each supplied with a 25 lambda bacteria suspension similar to that used in experiments of *V. anguillarum* #19264 (8.3×10^7/disc), *V. anguillarum* #19109 (7.3×10^6/disc), and *E. coli* (8.7×10^7/disc), all showed zones of agar digestion after 24 hr of incubation and much larger zones with longer incubation times. The zones of agar digestion (straw-colored areas surrounding discs in reddish-violet unaltered agar) appeared largest for *V. anguillarum* #19264, slightly less with #19109 and very small with *E. coli* (Fig. 6).

DISCUSSION

Little information is available on the ecological and physiological relationships that exist in mixed culture systems or natural environments. Myers *et al.* (*30*) and Mayer *et al.* (*29*) stated that the presence of bacteria had little effect on the culture productivity of *Chlorella pyrenoidosa*. Berland *et al.* (*8*) made the observation that several algal species grew much better in bacterized than in axenic culture and he concluded that growth-promoting vitamins were being released by the bacteria. Nakamura (*31*) showed that selected bacteria enhanced growth in

nutritionally deficient algal cultures and postulated that a true symbiotic relationship exists in nature. In another observation *P. tricornutum* grew better with low bacteria populations than in the absence of bacteria, but with high bacteria and low algae populations, algal growth was inhibited (*43*). The 2 marine bacteria isolates studied by Bell & Mitchell (*5*) had no effect on growth of *S. costatum*, *Cyclotella nana*, *Dunaliella tertiolecta*, and *Isochrysis galbana*, although growth of bacteria themselves was stimulated by the algae. Hence, although there are isolated reports of enhancement or lack of enhancement of algal growth by bacteria, there is little information on species-specific interactions in defined conditions.

Of particular interest in this investigation was the observation that *V. anguillarum* did not inhibit algal growth. *V. anguillarum* and many variant strains of the species have been described as the causative agent in diseases of finnock (*42*), eels (*6*), winter flounder (*24*), and salmon (*15*). The strain #19264 isolated from an ulcerous lesion in cod (*3*) was suggested as the "working type" species of *V. anguillarum* by ATCC, and strain #19109 was reported to cause bacillary necrosis in larvae and juvenile bivalve molluscs (*47*).

The production of physiologically significant amounts of the B vitamins, biotin and thiamine, and lesser amounts of B_{12} and nicotinic acid was demonstrated in many isolates of marine bacteria in pure culture (*11*). Although release of growth factors is a likely explanation for algal growth stimulation, other factors must also be considered, *eg*, solubilization, redox potential, buffering, CO_2 production, and excretion of inorganic phosphates, nitrates, or sulfates (*35*). The stimulation of *D. euchlora* by mixed bacterial cultures observed by McLachlan & Yentsch (*26*) was attributed to hydrolysis of beef extract and liberation of amino acids which can be utilized by this species (*16,20*).

Bell & Mitchell (*5*) found the term "phycosphere" useful in discussing bacterial-algal interrelationships. The term implies the existence of a zone of enhancement for a microbial population in proximity to the algae and that the microflora responds to the algal products by being attracted to this area. As a parallel term, "bactosphere" could be useful in describing the zone of enrichment surrounding bacterial populations that enhances and attracts other microorganisms. The "bactosphere" observed in these experiments that attracted algae appeared to be intimately associated with the living bacterial cell, was destroyed by heat or excessive drying, was not liberated extracellularly, was not associated with hydrolysis of complex organic nitrogen sources, could not be attributed to hydrolysis of paper, and could not be demonstrated in an artificial seawater medium. The tentative conclusion reached from these studies was that algal growth stimulation resulted from the digestion of agar by viable bacteria with the release of small molecules that stimulate algal growth. Other factors, such as CO_2, NO_3, PO_4 or SO_4 production by bacteria or physical-chemical conditions, cannot be ruled out completely. Since enhancement of algal growth in the presence of bacteria did not take place in artificial seawater medium, it does not argue for the importance of these latter factors. The bacterial populations, however, are probably not active in this inorganic medium so that these factors probably cannot be accurately evaluated.

The principal source of agar is from the genus *Gelidium* but it can also be extracted from other species with little change in properties from the different sources (*34*). Numerous marine bacteria possess the capacity to decompose agar, the majority are representatives of the *Vibrio-Pseudomonas* group (*44,51,52*). Agar consists of 2 polysaccharides, agarose (β-D-galactopyranose and 3,6-anhydro-L-galactopyranose residues) and agaropectin (an acidic polymere similar to agarose). Smaller amounts of D-galactose, L-galactose, D-xylose, and esters of sulfate glucuronic and pyruvic acids are also found (*2*). Some observations of algal growth stimulation with simple sugars have been reported, although, in general, such utilization is not widespread. Galactose utilization was observed in some chlorophytes (*32,41*) and in one unpublished study of marine phytoplankters (Ukeles & Rose), pyruvic acid stimulated growth of numerous species. These observations suggest that some product of agar hydrolysis, perhaps a sugar or acid, and maybe in a form not yet studied, stimulates algal growth.

In most cases the enhanced algal growth that appeared on the agar surface had the same gross appearance surrounding each bacterial disc. This observation suggests that the same factor is stimulating growth in all cases differing only in its concentration. The difference in extent of agar digestion between the 3 bacteria and the parallel prevalence of algal stimulation would support this contention. Certain responses, however, suggest that multiple factors are involved in enhancement of algae. The growth response to *V. anguillarum* #19264 by *D. euchlora* and *Pyramimonas* sp. was markedly different from other algae. In these algae, growth was pale in color, diffuse, and granular spreading out from the disc rather than dark in color, concentrated in and under the disc itself. There is also a suggestion of a very small zone of inhibition immediately surrounding the disc. It is also evident that for some species, *Pyramimonas*, *D. euchlora*, and *P. tricornutum*, the reaction of *E. coli* is as intense as to *V. anguillarum* #19264, although there is a minimum hydrolysis of agar with the *E. coli* strain. Answers to these questions will be sought in future work.

Stanier (*44*) cited the work of Bavendamm (*4*), who collected marine sediments near the Bahama

Islands that had 50,000–200,000 agar digesters/g, in commenting on the importance of agar digesters in the marine environment. The activities of these agar digesters could conceivably provide single or multiple factors that may be of significance in the promotion of algal blooms.

It appears self-evident that under natural conditions an intricate relationship must exist between bacteria and microalgae that is poorly understood and needs considerable study (*12,13,26*). This interaction is more complex than a simple competition for available nutrients. It is the authors' opinion that these interactions can best be elucidated by conducting controlled investigations in the laboratory.

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