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Test for Allelopathic Interactions Between Two Marine Microalgal Species Grown in Intensive Cultures

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Abstract. Both high pH and cell senescence are believed to lead to the production of toxic extracellular metabolites in freshwater microalgae. However, there was no evidence for allelopathic suppression of photosynthesis when filtrates of either of two marine microalgae, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, were mixed with whole cultures of the other species. This was true even when filtrate of *P. tricornutum* sample was derived from a culture at high pH or from one in various stages of senescence. It is believed that the major factor leading to the dominance of *P. tricornutum* in intensive outdoor cultures is the unique ability of this alga to tolerate pH levels above 9.5, not the allelopathic inhibition of competing species.

The ability of certain species of microalgae to excrete compounds that inhibit the growth of other algal species (allelopathy) is well established [13,22]. These compounds, although poorly defined, have in several cases been identified as long-chain fatty acids that are more soluble and, hence, more inhibitory at alkaline pH [16,20]. In addition, excretion of these metabolites seems to be enhanced when the stationary phase of growth is attained [19]. Sharp, Underhill, and Hughes [24] claimed that the marine diatom *Phaeodactylum tricornutum* Bohlin, which frequently dominates large-scale outdoor algal cultures, attains its competitive edge through allelopathic interactions. In contrast, we demonstrated that the dominance of *P. tricornutum* was due, in large part, to the alga's unique ability among marine microalgae to tolerate pH values above 9–9.5 [11,12]. In the current study we examined the possibilities that cell age and/or high pH enhance allelopathic interactions in the competition between *P. tricornutum* and the unicellular flagellated chlorophyte *Dunaliella tertiolecta* Butcher, which is a representative marine alga that cannot tolerate alkaline pH [11,12].

Materials and Methods

Test organisms. Cultures of both *Phaeodactylum tricornutum* Bohlin (clone TFX-1) and *Dunaliella tertiolecta* Butcher (clone Dun) were obtained from the culture collection of R. R. L. Guillard at the Woods Hole Oceanographic Institution.

Culture medium. Growth medium was enriched artificial seawater, as described previously [11].

Culture protocols and pH control. Both species were maintained in continuous culture at a dilution rate (medium flow rate/culture volume) of 0.5/day. The cultures were not axenic for reasons cited earlier [7]. The continuous culture apparatus consisting of two 0.5-liter cultures, the peristaltic feed pump (Model 1212, Harvard Apparatus Co., Inc., Millis, Massachusetts), and associated components, as well as the culture protocols, are described in detail elsewhere [2,11]. Continuous lighting (0.06 to 0.07 $cal \cdot cm^{-2} \cdot min^{-1}$) and temperature control (20°C) were maintained. Culture pH was controlled with a pH-stat system, as described previously [11], consisting of a combination pH probe mounted through a stopper at the top of the culture vessel and connected to a pH-controller (Model 650, Fisher Scientific Co., Pittsburgh, Pennsylvania). The controller activated a solenoid valve on a pressurized CO_2-enriched air line (1% CO_2) when desired pH was exceeded, allowing bubbled gas to enter the culture and lower the pH to the designated level. Fluctuations in pH did not exceed ±0.1 units from the set value.

Test for allelopathy. To test for possible allelopathic interactions, we carried out a series of short-term ^{14}C photosynthesis assays on mixtures of whole culture samples of one species and filtrates from cultures of other species. In each experiment comparisons of the rate of ^{14}C uptake were made between the samples containing mixtures of culture and filtrate and control samples consisting of mixtures of culture and filtered Sargasso Sea water, a relatively uncontaminated seawater [23]. All filtrates were obtained by filtering culture samples through glass fiber filters (Whatman GFF). The incubations lasted 30–35 min and were

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Table 1. Photosynthetic rates of two marine microalgal species (*Dunaliella tertiolecta* and *Phaeodactylum tricornutum*) when whole cultures of one species were diluted with mixtures of filtered seawater and filtrate of the other culture. Both the filtrate and whole culture sample were derived from steady-state continuous cultures.

Preparation		Relative mixture in assays[a]	Relative photosynthetic rate[b]	
Whole culture (pH)	Filtrate (pH)		Assay 1	Assay 2
D. tertiolecta (7.6)	*P. tricornutum* (7.6)	10:0:20[c]	1.00	1.00
		10:10:10	0.96	1.15
		10:20:0	ND[d]	1.19
D. tertiolecta (7.6)	*P. tricornutum* (9.0)	10:0:20[c]	ND	1.00
		10:10:10	ND	1.00
		10:20:0	ND	1.00
P. tricornutum (7.6)	*D. tertiolecta* (7.6)	10:0:20[c]	1.00	1.00
		10:10:10	0.81	0.96
		10:20:0	ND	0.88

[a] Whole culture:filtrate:seawater ratio by ml to equal 30-ml volume in assay vessel. Diluent seawater was Sargasso Sea water.
[b] The 2 assays are replicate experiments.
[c] Control assay containing only seawater as diluent.
[d] ND = not done.

initiated within 5 to 10 min after the samples were withdrawn from the continuous cultures to minimize any alterations in the physiological state of the algae or the chemistry of the filtrates.

When we harvested samples from the continuous culture vessels for use in the short-term assays we took additional samples for measurement of culture particulate carbon and nitrogen and cell number. Particulate carbon and nitrogen were analyzed with a C-H-N elemental analyzer (Model 240, Perkin Elmer, Norwalk, Connecticut) on samples retained on precombusted (550°C for 4 h) glass fiber filters (Whatman GFC). Cell counts were made with a hemacytometer (Spencer Bright-line, AO Instrument Co., Buffalo, New York).

Two types of assays were performed. The first assay involved mixing 10 ml of whole culture sample with 10 or 20 ml of culture filtrate obtained from steady-state continuous cultures. Filtered Sargasso Sea water was used as a control (no filtrate) or diluent to raise the total sample volume to 30 ml to maintain a constant algal concentration in each experiment. In all cases, whole culture samples were obtained from continuous cultures maintained at pH 7.6. Algal biomass in the continuous cultures was regulated by the combination of a fixed dilution rate and by supplying nutrients in excess so that light was growth limiting. The filtrates were taken from cultures maintained at pH 7.6 (*D. tertiolecta*) and pH 7.6 and 9.0 (*P. tricornutum*). To ensure that the incubation pH was stable at about 7.8 to 8.1, continuous culture of *P. tricornutum* at pH 9.0 was sparged with 1% CO_2 in air immediately before sampling to lower the pH to 7.6.

The second type of assay was similar to the first, except that the *P. tricornutum* culture first was switched from a continuous to a batch mode of growth. Then whole culture samples of *P. tricornutum*, taken periodically over a 10-day period, were mixed with either filtered seawater or filtrate from a steady-state culture of *D. tertiolecta* and assayed. Similarly, filtrates of the *P. tricornutum* were obtained over the 10 days, mixed with whole samples of *D. tertiolecta* from the continuous cultures, and assayed. Culture pH was kept at 7.6. For each experiment 10 ml of whole culture was mixed with either 20 ml of seawater (control) or 20 ml of filtrate.

We performed the ^{14}C incubations according to the procedure outlined by Li and Goldman [17]. Samples (30 ml) were placed in 150-ml water-jacketed glass incubation vessels (light intensity and temperature were identical to steady-state cultures) and $NaH^{14}CO_3$ added to give a final radioisotope concentration of 1.4–1.5μCi · μmole^{-1} total dissolved inorganic carbon. The samples were gently mixed with small magnetic stirring bars during the incubation. Whole-assay aliquots (1 ml) were harvested periodically over the course of the incubation and added to 2 ml methanol (acidified with 5% glacial acetic acid) in scintillation vials. The vial contents were evaporated to dryness under an infrared lamp and resuspended in 1 ml distilled water, followed by addition of 10 ml scintillation fluid (Handifluor). Radioactivity was measured by counting on a liquid scintillation spectrometer (Model LS-100C, Beckman Instruments, Inc., Fullerton, California). Appropriate quenching curves were established. Dissolved inorganic carbon was measured on a total carbon analyzer (Model PR-1, Dohrmann Envirotech, Santa Clara, California) according to the method of Goldman [8]. In all experiments, linearity in ^{14}C incorporation into algal biomass was attained over the incubation periods. Absolute photosynthetic rates of both the control samples (V_C) and the samples containing filtrate (V_F) were expressed as mg C · liter^{-1} · min^{-1} and were calculated from linear regression analysis of the photosynthesis curves. For all regression analyses, the r value (correlation coefficient) was >0.98, and the slopes were significant at the 0.001 level. Relative photosynthetic rates were the ratio of V_F to V_C.

Results

Addition of filtrate from steady-state cultures of either algal species had no discernible effect on the short-term photosynthetic rate of the other species (Table 1). For *Dunaliella tertiolecta*, this result occurred regardless of whether the pH of the filtrate-derived culture of *Phaeodactylum tricornutum* was 7.6 or 9.0 (Table 1). Similarly, when the growth mode was changed from continuous to

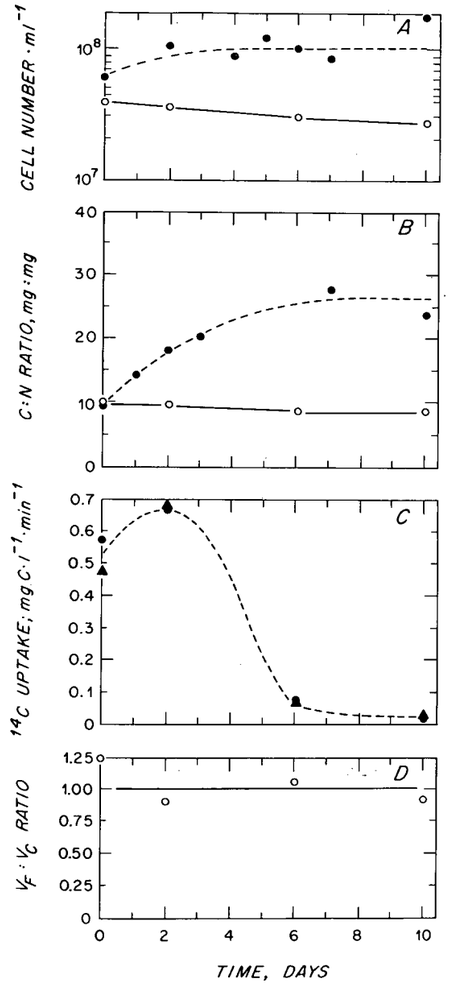

Fig. 1. Effect of cell age of *Phaeodactylum tricornutum* samples from culture switched from continuous to batch modes on allelopathic interactions with samples of *Dunaliella tertiolecta* from continuous culture maintained at dilution rate of 0.5/day. (A) Cell number, ●, *P. tricornutum* in batch mode; ○, *D. tertiolecta* in continuous mode. (B) Cellular C:N ratio, symbols same as in A. (C) Absolute photosynthetic rate of *P. tricornutum* during short-term assay, ●, V_F; ▲, V_C. (D) Relative photosynthetic rate of *D. tertiolecta* during short-term assay expressed as ratio of V_F to V_C.

batch, as reflected by the combination of increased cell number (Fig. 1A), increased cellular carbon to nitrogen ratio (Fig. 1B), and decreased absolute photosynthetic activity (Fig. 1C), the deteriorating physiological state of the *P. tricornutum* culture had no impact on the relative photosynthetic rate of *D. tertiolecta* (Fig. 1D). The relative photosynthetic activity of *P. tricornutum* likewise was unaffected by the addition of *D. tertiolecta* filtrate, even though the absolute photosynthetic rate of the diatom diminished tremendously after two days in the batch mode (Fig. 1C).

Discussion

High pH and/or increased cell age are known to stimulate allelopathy among numerous freshwater algae [16,19,20], yet we found no evidence that either of these conditions, even extreme senescence (Fig. 1), led to the production of toxic extracellular metabolites by *Phaeodactylum tricornutum*. Although not measured, the release of extracellular metabolites most likely increased after the continuous culture of *P. tricornutum* was switched to a batch mode and the cellular carbon to nitrogen ratio increased to 25 (Fig. 1B); still, the filtrate of the *P. tricornutum* culture did not inhibit photosynthesis in *Dunaliella tertiolecta* (Fig. 1D).

Our results generally are consistent with those of D'Elia, Guillard, and Nelson [5], who found no evidence for allelopathy when the marine diatom *Thalassiosira pseudonana* Hasle and Heimdal (3H) was exposed to filtrate from a late-exponential-phase culture of *P. tricornutum*. In contrast, Sharp, Underhill, and Hughes [24] found that cell death leading to culture washout ensued when steady-state continuous cultures of *T. pseudonana* (3H) were exposed to whole cells of *P. tricornutum* that originated from a stationary-phase culture. Similarly, Chan et al. [3], using an algal plating technique, found that filtrate from stationary-phase cultures of three out of four clones of *P. tricornutum* suppressed the growth of the marine diatom *Cylindrotheca fusiformis* Reimann and Lewin. The one clone (Pet Pd.) that did not inhibit growth of *C. fusiformis* was the same clone used by both Sharp, Underhill, and Hughes [24] and D'Elia, Guillard, and Nelson [5] in their allelopathy studies that yielded opposing results. Moreover, the toxic TPH 77 clone used by Chan et al. [3], and the nontoxic TFX-1 clone used in this study all were isolated at different times from enriched natural seawater that originated from Cape Cod, Massachusetts, coastal waters; thus without evidence to the contrary, these clones may indeed have similar physiological characteristics, thereby making it impossible to recon-

cile on the basis of the data available the reported differences in allelopathic potential among these isolates.

In spite of this, for several reasons it does not seem tenable that *P. tricornutum* gains its competitive edge in intensive outdoor cultures through allelopathy, as suggested by Sharp, Underhill, and Hughes [24]. First, even though we found no evidence for the production of toxic extracellular metabolites by *P. tricornutum* at any pH tested, we showed earlier that *P. tricornutum* outcompetes *D. tertiolecta* most effectively at pH values >9 [12]. Second, the inability of *D. tertiolecta* to grow when the pH exceeds 9 to 9.5 [11,12] is characteristic of many marine microalgae, including *Thalassiosira pseudonana* (3H) [6,21]. Thus, intense growth in poorly buffered cultures can lead to high pH, which, in turn, would prevent the growth of pH-sensitive species and allow *P. tricornutum* to grow unrestricted [12]. This point is dramatized by the fact that when *P. tricornutum* dominates outdoor mass cultures, the pH typically is >10 [4,9,10]. Finally, even if senescence is a prerequisite for the excretion of toxic metabolites by species such as *P. tricornutum*, allelopathy should not be a factor in influencing the growth of competing species in mass cultures because such cultures usually are maintained in the exponential phase [9].

A common problem in testing for allelopathy is the difficulty in rigidly controlling the experiment to avoid introducing subtle changes in growth conditions (e.g., changes in pH, nutrient availability) when whole cultures of one species are mixed with filtrates or whole cultures of another. For example, because culture pH was not regulated in any of the previous studies that dealt with allelopathy in *P. tricornutum* [3,5,24], drastic reductions in growth of the test algae caused by even slight increases in pH could have been mistaken for allelopathic inhibition. Similarly, without rigid control of growth rate and the physiological state of the cell population, it is difficult to establish cause-and-effect relationships that describe allelopathic interactions between competing species.

A role for allelopathy in competition among microalgae in natural fresh waters seems firmly established [22]. Keating [14,15], argues that allelopathy is a major factor contributing to the success of blue-green algae in highly eutrophic fresh waters. Furthermore, Murphy, Lean, and Nalewajko [18] suggest that excretion of hydroxamate chelators by blue-green algae during periods of iron unavailability either enhances their own growth or inhibits the growth of competing species. The success of *P. tricornutum* at high pH may be due to a similar excretion phenomenon in which the availability of sparingly soluble trace metals and other nutrients is sustained by the production of extracellular chelators [1]. However, the role of allelopathy in competitive interactions between marine microalgae in healthy, intensive cultures is poorly defined [25].

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