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22. The composition of the Ringer solution was 120 m*M* NaCl, 2.5 m*M* $KHCO_3$, and 2 m*M* $CaCl_2$, adjusted to *p*H 7.6.
23. The composition of the Ca-free Ringer solution was as given in (*22*) except that 1 m*M* EDTA was used in place of the $CaCl_2$. The muscle bath was flushed three times at each solution change.
24. It is necessary to show not only that the initial concentration of TTX is sufficient to saturate these receptor sites, but also that uptake of TTX by these high-affinity receptors does not significantly alter the initial TTX concentration. The total binding (specific plus nonspecific) reported by Almers and Levinson (*18*) indicates that a maximum 5 percent concentration change would occur under the experimental conditions of this study.
25. The preparation was photoirradiated for five 1-minute periods, at 1-minute intervals, to avoid heating the muscle tissue. Two tungsten halogen projector lamps (650 watts, DVY, 3400°K) were situated 10 cm to either side of the preparation bath but slightly above the fluid level to ensure direct illumination of the submerged muscle. Cooling was supplied as necessary to maintain the bathing medium at room temperature (22°C).
26. Although TTX-AβA$_2$ also showed TTX activity, the activity was not maintained when the preparation was washed after photoirradiation. It seems reasonable to suppose that in this analog the stereochemical positioning of the arylazido photoactive ligand on the TTX molecule prevented its covalent insertion during photoirradiation.
27. We thank S. J. Jeng for her helpful assistance in the preparation of the arylazido-β-alanine tetrodotoxin analogs and T. Kosaki for his assistance in the measurements of muscle membrane potential. Supported in part by grants from the American Heart Association (R.H.G.) and by NSF grants PCM76-02974 (R.J.G.) and BNS76-02647 (J.S.D.).

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Allelopathic Influence on Blue-Green Bloom Sequence in a Eutrophic Lake

Abstract. *The bloom sequence in a eutrophic lake, Linsley Pond, over a period of 3 years is correlated to the effects of cell-free filtrates of dominant blue-green algae on both their successors and their predecessors. There is unbroken correspondence between the effects of heat-labile probiotic and antibiotic filtrates and the rise and fall of bloom populations in situ. All organisms in vitro were axenic or unialgal (bacterized) isolates from Linsley Pond.*

When for fiscal or logistic reasons excess nutrients cannot be eliminated from freshwater lakes, certain species of phytoplankton grow to excess. Such species might be better controlled if we could obtain a clearer understanding of bloom sequence; that is, of which phytoplankter will grow to excess, and when.

Bloom sequence in eutrophic freshwater lakes is the overt expression of multiple, interacting factors, and any phytoplankter which produces a bloom population is one which is momentarily most favored by the summation of the effects of these factors. Such effects function as either coarse or fine adjustors of the natural bloom sequence. Coarse adjustors, such as light (*1*) and macronutrients (*2*), determine the production capacity of the system and, to a degree, the major categories of dominant organisms; for example, diatom blooms reflect silicon availability (*3*). In contrast, fine adjustors, such as micronutrients (*4*) and variations in temperature patterns (*5*), determine the specific organisms of dominance. No fine adjustor, however, has been identified which accounts for bloom sequence. Rather, the best examples presented account for single instances of dominance (*6*).

Since the early 1900's (*7*), the effects of extracellular metabolites (allelopathy, probiosis, and antibiosis) have been suggested as playing a major role in sequence determination, and decades of speculation and investigation have followed (*8*). There are many reports of interactions occurring between growing organisms in vitro (*9*). Such interactions are frequently attributed to the effects of extracellular metabolites. Although many of the reported effects can be assigned to direct competition for nutrients or to other nonmetabolite influences, several authors have presented evidence that algal metabolites are both suitable in quality and sufficient in quantity to account for reported interactions (*6, 10*). Yet, extrapolation of such data from experiments in vitro to explanations of dominance in situ is universally challenged. The most severe criticisms concern either the differences in the dilutions of active metabolites in vitro and in situ, or the relationships of study organisms to each other or to any natural community. The advisability of extrapolating quantitative information is specifically questioned because population densities in vitro have far exceeded those in situ. It has been suggested that in a lake such factors as *p*H or nutrients could totally mask an allelopathic response observed in vitro only because of the increased concentration of the toxic plant product under laboratory conditions (*11*). Qualitative extrapolation is similarly challenged because (i) species are used which do not occur together in nature (organisms in tests are isolated from different locales, often from a convenient culture collection), and (ii) bacterial activity is not taken into account.

In consideration of these criticisms certain procedures were established for this study. All organisms were isolated from the natural community of a single, eutrophied lake—Linsley Pond, North Branford, Connecticut. Population densities in metabolite-producing (producer) cultures (*12*) were monitored and were found similar to those of epilimnetic blooms in Linsley. This similarity reflects both the high population densities in Linsley and the composition of growth media. The basic growth medium in producer cultures consisted of equal volumes of separately aged and charcoal-treated water samples obtained from Linsley before and after the fall turnover (*13*). Macronutrient additions (*14*), treatment of the cultures (*15*), and inocula for producer and test cultures were identical, and all *p*H values were within a range of 0.1 unit for any given set of tests (*16*) and control (*17*) cultures. The only difference between treatments of test and control cultures was the autoclaving of control cultures prior to enrichment, *p*H check, and inoculation with bioassay organisms.

Although examples of heat-stable allelopathic effects were noted, only heat-labile effects are included in this report (*18*). For *Anabaena holsaticum* the heat-labile substance was removed from filtrates by ultrafiltration, by dialysis, and by ether extraction. In each instance the substance was active when returned to bioassay cultures at its natural concentration, or at two or five times the natural concentrations. Cultures diluted to 0.5 times also retained activity.

Table 1 shows a summary of the probiotic and antibiotic effects of axenic, cell-free filtrates of each bloom-dominant phytoplankter on organisms which bloomed before or after it. Filtrates of each dominant species produced only negative, or neutral, effects on its imme-

Table 1. Effects of filtrates of dominant blue-green algae on predecessors and successors of dominant species (1971 to 1974). Numbers in parentheses are culture collection identification numbers and are used in place of Latin names for brevity. Symbols: +, enhancing effect; −, inhibiting effect; 0, no effect observed.

Dominant alga	Condition of culture	Period of dominance (months)	Predecessors	Effect of dominant on predecessors	Successors	Effect of dominant on successors	Contemporaries	Effect of dominant on contemporaries	Period of co-occurrence
Oscillatoria rubescens (535)	Bacterized	NDJFMA			(739)	0			
					(538)	+			
Oscillatoria agardhi (739)	Bacterized	AMJJ	(535)	0	(538)	+	(538)	+	Entire 538 bloom
					(762)	*			
					(535)	0			
Anabaena holsaticum (538)	Axenic	Jun	(739)	−	(762)	*	(739)	−	End of 739 bloom
					(535)	+			
Aphanizomenon elenkinii (762)	Bacterized	JAS	(739)	−			(535)	−	End of 535 bloom
Oscillatoria rubescens (535)	Bacterized	JAS (O)	(739)	0	(597)	+	(765)	−	Rapid end of 765 bloom
					(776)	+			
							(762)	*	
							(597)	+	Start of 597 bloom
Pseudanabaena galeata (597)	Axenic	O	(535)	0	(776)	0	(776)	0	
Oscillatoria sp. (776)	Axenic	OND (J)	(535)	−			(597)	−	End of 597 bloom
			(597)	−					
Synechecoccus sp. (91)	Axenic	AM			(765)	+			
Anabaena sp. (765)	Bacterized	JJA					(762)	*	
Aphanizomenon elenkinii (762)	Bacterized	A	(765)	−	(7 months free of blue-green blooms)		(765)	−	End of 765 bloom

**Aphanizomenon elenkinii* (762) was not included among the assay organisms; therefore, no data are available for this species.

diate predecessors (*19*). In contrast, filtrates of these same dominant species produced only positive, or neutral, effects on their immediate successors (*20*). Both patterns of interaction enhance the competitive position of that organism which will dominate an impending bloom.

To strengthen the extrapolation of the data obtained from cultures to the explanation of phenomena in situ, additional bioassays were conducted with water samples freshly collected from Linsley Pond at times considered significant to the bloom pattern; that is, before, during, or after bloom maxima. Some of these samples which could not be assayed immediately on collection, were stored (axenic and frozen) and examined later (*21*). The heat-labile effects in these samples correlate well with data obtained from filtrate studies and with sequence in situ.

Since each of the annual sequence patterns was distinct, a cross-comparison of the allelopathic effects of organisms that grew to excess one year but did not do so in the next year was undertaken. The results showed that the allelopathic effects in vitro corresponded well with the sequence pattern in situ.

The consistent long-term agreement of allelopathic effects and bloom sequence, while supportive, is not essential to the contention that allelopathic effects are a significant factor in bloom sequence determination. It is compatible with this premise that allelopathic effects alone could produce a pattern of sequence quite different from that observed. While such inconsistencies were not encountered in the present study, they could nevertheless be common. In that hypothetical situation the summation of the effects of the multiple, interacting factors which control sequence (light, nutrients, predation, and allelopathy) would not be identical in direction to the single effect of allelopathy. This would result in an overriding of allelopathic influences. It is essential to reiterate, however, that this 3-year pattern did not show such contradictions. This lack of contradiction must be interpreted as a reasonable basis for the contention that allelopathy, in its many forms, is at least one of the most significant of the fine adjustors in bloom sequence determination.

The implication of allelopathy as a major controlling factor in bloom sequence determination presents a variety of challenging alternatives for future research and development. Application of such information toward the prediction of algal bloom sequence would allow limitation of the duration and density of offensive bloom populations by sequence manipulation. This, then, would offer immediate and practical lake management potential. Such a predictive capacity awaits a clearer understanding of some of the mechanisms underlying demonstrable allelopathic effects and, in some instances, may require biochemical identification of the specific compounds involved. While such identification of natural products is often difficult, it is unusually desirable in this case because it would instigate the development of ecologically desirable, highly specific, biological algacides. The application of such biologically based algacides in lieu of the ubiquitous, and undesirable, application of copper sulfate is analogous to the use of biological insect controls in lieu of broad spectrum insecticides.

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16. Test cultures were 10-ml portions of the cell-free filtrate of a producer culture, enriched with macronutrients by the addition of 1½ percent enrichment seawater I (*14*), and inoculated with the assay organism (that is, with a predecessor or successor of the dominant organism).
17. Control cultures were identical to test cultures except that the controls were autoclaved after filtration and before macronutrient addition.
18. Additional effects are considered in (*13*). The few heat-stable effects noted were consistent with bloom sequence; however, since such effects were not sought, there are probably many additional heat-stable effects which were not noted and whose effects cannot be properly considered in this study.
19. Because the allelopathic effects of a dominant species cannot occur prior to that species occurring at density, the term predecessor is limited to bloom organisms whose growth at least minimally overlaps the dominant species in question.
20. The term successor includes blooms which occurred within approximately 2 weeks of the dominant in question.
21. Because freezing sometimes produced changes in heat-labile effects, generally decreasing them, these data are considered less reliable than the others.
22. I thank L. Provasoli and G. E. Hutchinson for guidance; R. Patrick, S. Golubic, and F. Drouet for assistance with taxonomic questions; Haskins Laboratories, Yale University, and the Department of Environmental Science, Rutgers University, for support. This work was supported in part by EPA research grant RA 801387.

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Light and Stomatal Function: Blue Light Stimulates Swelling of Guard Cell Protoplasts

Abstract. *Onion guard cell protoplasts swell when illuminated with blue light. The response is a 35 to 60 percent increase in volume and is dependent on potassium ion. Epidermal cell protoplasts do not swell under the same conditions. It is postulated that a membrane-bound blue photoreceptor mediates a direct response of guard cells to light.*

Protoplasts isolated from onion guard cells by enzymatic digestion swell when illuminated with broadband blue light (peak at 410 nm). Evidence suggests that the response, which is K^+-dependent, is a manifestation of normal guard cell activities. We envision the light-stimulated mechanism as an activation of a membrane-bound electron transport chain that results in an influx of K^+ into the cell. The resulting decrease in water potential mediates an influx of water, which leads to an increase in protoplast volume.

Guard cells surround the stomatal pores on the leaf epidermis of higher plants. They control the gas and water content of the leaf by swelling and contracting, thus respectively opening and closing the stomata. Light induces opening of intact stomata (*1*, *2*). Previous studies on different species have shown that the blue, red, and far-red regions of the spectrum are effective, with blue being the most active on a quantum basis. Chlorophyll has been implicated as the photoreceptor because of the similarity between its absorbing properties and the action spectrum of stomatal opening (*1*, *2*). However, because that view fails to explain the greater sensitivity of stomata to blue light (*3*), it is possible that other pigments play a role.

The mechanism by which light affects stomatal action remains controversial. There is substantial evidence that light is sufficient (*4*, *5*) but not necessary for stomatal opening, since, in the dark, stomata open in low concentrations of CO_2 and close in high concentrations (*6*). It is most commonly accepted that all light responses are indirect and actually mediated by changes in the concentrations of CO_2 resulting from photosynthesis (*1*, *2*, *7*). However, the possibility that guard cells might have specific mechanisms that respond directly to light or that both light and CO_2 can act directly on the same receptor deserves consideration. Changes in turgor pressure that cause stomatal opening and closing are mediated by massive transport of K^+ to and from the guard cells (*7*); it thus seems possible that light, which is known to drive ion movements in several systems (*8*), might directly stimulate a similar membrane transport phenomenon in guard cells. Our findings, as well as many known features of stomatal action, seem best accounted for if light is a primary activator.

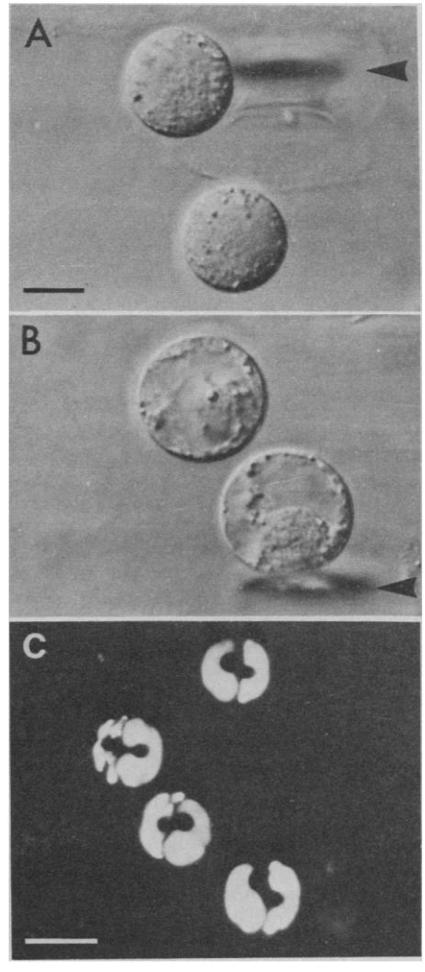

Fig. 1 (A and B). Photomicrographs of protoplasts from onion guard cells. Arrows indicate the undigested stomatal ridges that surround the pores. Scale bar, 10 μm. (A) Typical protoplasts in 0.45*M* mannitol and 0.5 m*M* $CaCl_2$, unexposed to light, showing a dense, granular cytoplasm. (B) Typical swollen protoplasts after a light treatment. A prominent vacuole occupies most of the cell volume. (C) Photomicrograph of an onion epidermal peel seen under dark-field fluorescence microscopy. Scale bar, 40 μm. An excitation filter (Reichert BG12) (peak at 410 nm) and a barrier filter (Reichert Sp3) with a 50 percent cut-off at 500 nm were used. The guard cells but not the neighboring epidermal cells fluoresce in the green. The cell wall and the nucleus are opaque.

Guard cell protoplasts are prepared in microchambers by digesting thin paradermal slices from 7- to 10-day-old onion cotyledons with 4 percent (weight/volume) Cellulysin in a 0.23*M* mannitol solution (*9*). After 8 to 14 hours of digestion, the spherical protoplasts (Fig. 1A) can easily be recognized because (i) they are smaller than epidermal or mesophyll protoplasts and lack visible chloroplasts (*10*); (ii) they display a characteristic dense, granular cytoplasm with little evidence of a vacuole; and (iii) they usually remain in the vicinity of the undigested ridge that surrounds the pore. Guard cell protoplasts kept in mannitol in the dark stream actively for several hours. However, they swell steadily and finally burst. This "dark" swelling is usually reduced by adding 0.5 m*M* $CaCl_2$ to the medium. Only preparations with protoplasts averaging less than 23 μm in diameter were used in the experiments.

A typical experiment included an overnight enzyme digestion followed by a wash with 0.3 to 0.5*M* mannitol plus 0.5 m*M* $CaCl_2$. The chambers were left in the dark for 1 to 3 hours, and a solution of 0.32*M* mannitol, 0.5 m*M* $CaCl_2$ and 30 m*M* KCl was introduced just before the measurements of protoplast diameter. Measurements were made with a split-image eyepiece (Vikers) operated at ×150 to ×375 with a green interference filter transmitting at 546 nm. Protoplasts