

## THE EFFECT OF pH IN INTENSIVE MICROALGAL CULTURES. I. BIOMASS REGULATION<sup>1</sup>

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**Abstract:** Two freshwater and two marine algal species were grown in intensive continuous cultures at a fixed dilution rate of  $0.5 \cdot \text{day}^{-1}$ , but at varying pH levels in the range 7.6 to 10.6. Both freshwater species, *Scenedesmus obliquus* (Turp.) Kutz. and *Chlorella vulgaris* Beij., grew up to pH 10.6 although *C. vulgaris* was more adversely affected by alkaline pH than was *Scenedesmus obliquus*. Of the marine species, *Phaeodactylum tricornerutum* (TFX-1) Bohlin was hardly affected by varying pH up to its maximum tolerable level of 10.3, whereas growth of *Dunaliella tertiolecta* (Dun) Butcher was adversely affected by increasing pH and ceased when the pH exceeded 9.3. These results are consistent with the general observations that many marine species cannot tolerate alkaline pH values much above 9.5. Moreover, the unique ability of *Phaeodactylum tricornerutum* to grow at pH >10 probably is a major factor contributing to its well documented success in large-scale outdoor cultures that are poorly buffered. It is difficult to separate metabolic from purely chemical factors that influence the pH tolerance limits of the individual species. The lower pH limits were, however, distinctly controlled by the production of alkalinity concomitant with  $\text{NO}_3^-$  uptake, whereas the upper pH limits in the case of *Scenedesmus obliquus* and *Phaeodactylum tricornerutum* seemed to be regulated primarily by metabolic control. In no case was the availability of inorganic carbon an influencing factor in setting the maximum attained pH.

### INTRODUCTION

Attempts to maximize phytoplankton biomass yields via the supply of excess nutrients while still maintaining desired species in culture have met with varying degrees of success (Goldman, 1979). For example, a common problem has been the infestation and rapid takeover of non-axenic mass cultures of desired species by weed microalgae such as the chlorophyte *Scenedesmus* sp. in freshwater systems and the marine diatom *Phaeodactylum tricornerutum* in marine counterparts (Oswald & Golueke, 1968; Goldman & Ryther, 1976). Goldman (1976) speculated that among a group of marine species possessing similar growth characteristics in intensive culture, *P. tricornerutum* often is the successful competitor because of its ability to tolerate high pH or excrete toxic compounds (allelopathy) or both; as shown by Goldman & Ryther (1976), however, this competitive edge occurs only within a restricted temperature range of  $\approx 10$  to  $20^\circ\text{C}$ .

To date, the rôle of allelopathy in competition between *P. tricornerutum* and other marine algae is poorly defined. On the one hand, Sharp *et al.* (1979) claimed that

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the ability of *P. tricorutum* to outcompete another diatom, *Thalassiosira pseudonana* (3H) was due to allelopathy. In contrast, D'Elia *et al.* (1979) and Nelson *et al.* (1979) suggested that, rather than any allelopathic interaction, the outcome of competition between these two species was determined by the ability of *Phaeodactylum tricorutum* to outgrow *Thalassiosira pseudonana* (3H) at the very low light intensities that prevail in dense cultures.

In searching for other possible causes of this competitive interaction, we discovered from the literature that virtually in every case in which *Phaeodactylum tricorutum* dominated in mixed cultures the pH was not controlled; moreover, in those studies which contained data on pH (Goldman & Ryther, 1975; D'Elia *et al.*, 1977) or where such data were available but unreported (Goldman, unpubl. data), the pH was found to rise above 10 when *P. tricorutum* became the major species. In addition, one of us (Goldman, 1976) observed that the highest pH attained in continuous monocultures of *P. tricorutum* consistently was  $>10$ , whereas in similar cultures of *Thalassiosira pseudonana* (3H) and *Dunaliella tertiolecta* it did not exceed  $\approx 9.4$ .

These results were consistent with the findings of Humphrey (1975), who showed that *Phaeodactylum tricorutum* was among a small group of marine algae that could tolerate pH values  $>10$ . Hence, we concluded that there was a strong circumstantial argument in support of a rôle for pH control over species dominance and that further research on this topic was necessary. In Part I of the current study we examine the rôle of pH on biomass regulation in intensive cultures of both marine and freshwater microalgae, independent of inorganic carbon limitation. In Part II we shall investigate the impact of pH on species competition (Goldman *et al.*, 1982).

## MATERIALS AND METHODS

### TEST ALGAE

We obtained cultures of *Phaeodactylum tricorutum* (TFX-1) Bohlin and *Dunaliella tertiolecta* (Dun) Butcher from the collection of R. R. L. Guillard at the Woods Hole Oceanographic Institution for use in the marine studies. We chose the latter species in preference to *Thalassiosira pseudonana* (3H) for the competition studies with *Phaeodactylum tricorutum* mainly to avoid any possibility that silicon limitation could bias the results. Both *P. tricorutum* and *Dunaliella tertiolecta* do not require silicon, whereas *Thalassiosira pseudonana* has a well-defined requirement for this nutrient (D'Elia *et al.*, 1979).

The green chlorophytes *Scenedesmus obliquus* (Turp.) Kutz. and *Chlorella vulgaris* Beij. were used in the freshwater studies and came from the laboratory of M. Gibbs at Brandeis University.

## NUTRIENT MEDIA

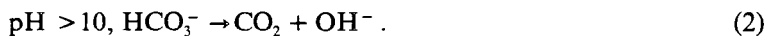
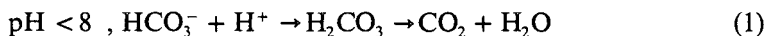
Medium for the marine studies was synthetic sea water containing 400 mM NaCl, 20 mM MgCl<sub>2</sub>, 20 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, 10 mM KCl, 0.8 mM KBr, 0.2 mM H<sub>3</sub>BO<sub>3</sub>, 2 mM NaHCO<sub>3</sub>, 5 mM NaNO<sub>3</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, and vitamins and trace metals plus the sodium iron salt of ethylenediaminetetraacetic acid (EDTA) in a two-fold dilution of the amount in *f*-medium (Guillard & Ryther, 1962).

The freshwater medium contained 0.4 mM MgCl<sub>2</sub>, 0.4 mM MgSO<sub>4</sub>, 0.2 mM CaCl<sub>2</sub>, 2 mM NaHCO<sub>3</sub>, 12 mM NaNO<sub>3</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub> and the same concentrations of EDTA-trace metals as in the sea-water medium.

## CULTURE SYSTEM AND pH CONTROL

The continuous-culture apparatus (a bank of seven 0.5-l cultures), the culturing protocols, and the experimental analyses virtually were identical to those described previously (Goldman *et al.*, 1981). Continuous lighting (0.06–0.07 cal · cm<sup>-2</sup> · min<sup>-1</sup>), temperature control (20°C), and mixing with Teflon-coated stirring bars were employed in all experiments. Liquid medium was metered into the cultures at a fixed dilution rate *D* (medium flow rate/culture volume) of about 0.45–0.60 · day<sup>-1</sup> via a multi-channel peristaltic pump. Both the medium and the peristaltic pump were housed in a refrigerator maintained at 5°C. Medium tubing was constructed of glass and Teflon with a small section of silicon tubing inserted through the pump.

We maintained the culture pH at various levels in the range 7.6–10.6 with a pH-stat system, identical in design to those of Soltero & Lee (1967) and Sperling *et al.* (1974), that provided sufficient CO<sub>2</sub>-enriched air (1% CO<sub>2</sub>) to balance photosynthetic uptake of inorganic carbon. The system consisted of a combination pH probe mounted through a stopper at the top of the culture vessel and connected to a pH-controller (Fisher model 650). The controller activated a solenoid valve on a pressurized CO<sub>2</sub>-enriched line when a desired pH was exceeded; bubbled gas then entered the culture through a port at the base of the culture, lowering the pH to the designated level. Fluctuations in pH did not exceed ± 0.1 U from the set value. At the start of an experiment when algal biomass was increasing to a steady-state level, the pH rose to the designated level because inorganic carbon was provided solely from bicarbonate alkalinity via the following reactions:



Between pH 8 and 10 (the region in which most of the experiments were performed) both reactions (1) and (2) are important (Kern, 1960). Once the designated pH was attained, any additional inorganic carbon requirements were met by the flow of bubbled CO<sub>2</sub>, as regulated by the pH controller. In this manner, we ensured that inorganic carbon was not limiting at any pH level tested because there was no

restriction on the amount of inorganic carbon supplied – as long as there was a photosynthetic requirement for inorganic carbon this demand was always met by the introduction of gaseous  $\text{CO}_2$ .

We obtained a mixture of 1%  $\text{CO}_2$  in air by blending 100%  $\text{CO}_2$  from a pressurized cylinder with laboratory air in a two-gas proportioner. The flow rate of gas to each culture was held constant at  $0.71 \cdot \text{min}^{-1}$  with a rotometer on the gas inlet. The total amount of  $\text{CO}_2$  supplied to each culture was monitored by wiring a clock to the solenoid valve and recording the time the valve was open. A schematic view of one of the six pH-stat systems is shown in Fig. 1. We operated a seventh unit without pH regulation by bubbling air (0.036%  $\text{CO}_2$ ) into the culture at a rate of  $0.71 \cdot \text{min}^{-1}$ . In this fashion culture pH rose to a level determined by algal growth and its concomitant effect on the  $\text{CO}_2$ - $\text{HCO}_3^-$ - $\text{CO}_3^{2-}$  chemical system (Goldman *et al.*, 1972).

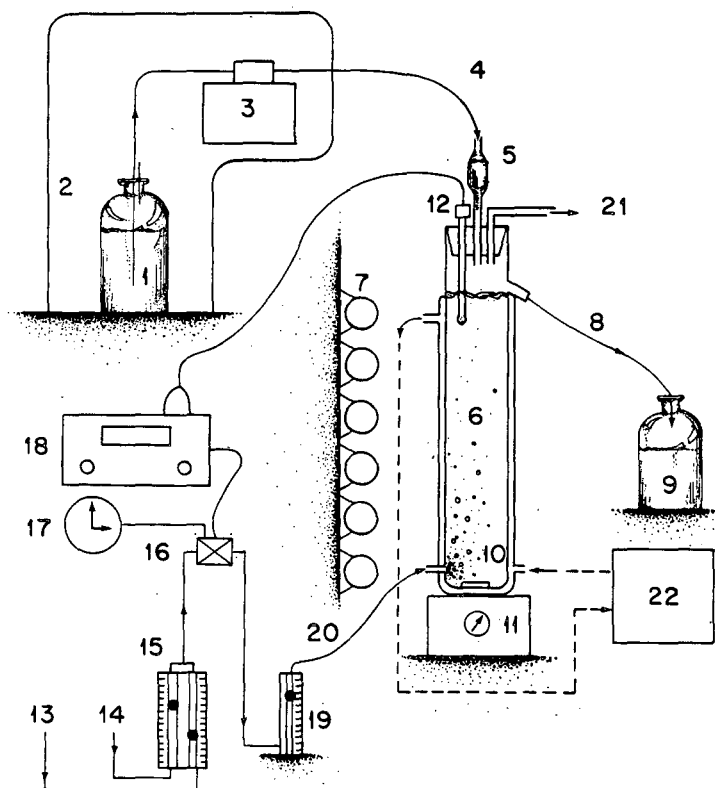


Fig. 1. Schematic view of continuous culture and pH-stat system: 1, medium supply bottle; 2, refrigerator; 3, multi-channel peristaltic pump; 4, medium supply line; 5, siphon-break; 6, 0.5-l culture vessel; 7, bank of fluorescent lights; 8, culture overflow line; 9, culture overflow bottle; 10, magnetic stirring bar; 11, magnetic stirrer; 12, combination pH probe; 13, laboratory air supply; 14, 100%  $\text{CO}_2$  supply; 15, two-gas proportioner; 16, solenoid valve; 17, clock timer; 18, pH-controller; 19, gas flow meter; 20, influent gas line; 21, effluent gas line; 22, recirculating temperature control bath.

## CULTURE OPERATION

We performed four sets of experiments, one with each of the test species. Each experiment consisted of establishing steady state levels of biomass at designated pH levels in the range 7.6 to 10.6. When steady state was reached the cultures were sampled for cell counts, particulate carbon, and particulate nitrogen.

## ANALYSES

Particulate carbon and particulate nitrogen were measured on a Perkin Elmer 240 elemental analyzer. Cell counts were made on a Spencer Bright-line hemocytometer. Because steady-state growth rates  $\mu$  were equal to  $D$ , the maximum tolerable pH for each species was defined as the pH above which  $\mu$  could not be sustained at  $0.5 \cdot \text{day}^{-1}$  and cell washout ensued.

## RESULTS AND DISCUSSION

### LOWER pH LIMITS

We found that it was impossible to establish pH values  $< 7.6$  in the marine growth medium and 7.9 in the freshwater medium (Table I). Based on established pH tolerances of our test species (see Emerson & Green, 1938; Osterlind, 1949; Hayward, 1968; Humphrey, 1975), our inability to attain steady-state pH levels below these lower limits was not due to a physiological limitation, but rather represented the respective equilibrium pH values that resulted when the partial pressures of  $\text{CO}_2$  in the gas and liquid phases came into equilibrium. The equilibrium pH is controlled, not only by the partial pressure of  $\text{CO}_2$  in the gas phase, which in this case was 0.01 atm, but also by the alkalinity (Stumm & Morgan, 1970). In the present experiments the alkalinity was variable and increased above the initially added 2 meq  $\text{HCO}_3^-$  in direct proportion to the amount of  $\text{NO}_3^-$  assimilated by the algal cultures (Osterlind, 1949; Brewer & Goldman, 1976). Hence, when the pH controller was set for a pH lower than the equilibrium value, the solenoid valve always remained open and 1%  $\text{CO}_2$ -enriched gas was supplied continuously at a flow rate of  $0.71 \cdot \text{min}^{-1}$ . This influx of inorganic carbon was in great excess relative to the growth requirements of the algal cultures (Goldman *et al.*, 1981), so that  $\text{CO}_2$  equilibrium between the gas and liquid phases was attained. The pH under the above conditions was determined indirectly by the steady state concentration of algae because alkalinity increase was proportional to biomass production.

TABLE I

Summary of maximum tolerable pH and average cellular chemical composition data.

Species	pH range		Algal biomass at equilibrium pH (mg C · l <sup>-1</sup> ) <sup>c</sup>	Cell carbon (pg · cell <sup>-1</sup> )	Carbon : nitrogen ratio (mg : mg)
	Equilibrium <sup>a</sup>	Maximum <sup>b</sup>			
<i>Phaeodactylum tricornutum</i>	7.6	10.3	≈ 500	14.8 ± 1.93 <sup>d</sup>	6.5 ± 0.82 <sup>d</sup>
<i>Dunaliella tertiolecta</i>	7.6	9.4	350	21.8 ± 1.66	5.5 ± 0.31
<i>Scenedesmus obliquus</i>	7.9	10.6	750	24.1 ± 5.34	6.1 ± 0.20
<i>Chlorella vulgaris</i>	7.9	10.6	650	6.5 ± 1.15	5.0 ± 0.44

<sup>a</sup> Minimum pH established by combination of increase in alkalinity and 1% CO<sub>2</sub>.

<sup>b</sup> Maximum pH for which a steady-state dilution rate of 0.5 · day<sup>-1</sup> could be maintained.

<sup>c</sup> Steady-state algal biomass at dilution rate of 0.5 · day<sup>-1</sup>.

<sup>d</sup> Mean ± SD from all pH experiments; all values were significant at the <0.01 level.

## UPPER pH LIMITS

From previous experience (Goldman *et al.*, 1981), we found that light limitation in our culture system could be attained with the freshwater algae by providing 12 mM inorganic N and 1 mM P. Growth of the marine algae under these conditions was erratic and the cultures tended to clump, particularly at the higher pH values. Hence, we were forced to reduce the  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  concentrations in the marine growth medium to 5 mM and 0.5 mM, respectively to ensure culture stability, but at the expense of allowing the cultures to become depleted in  $\text{NO}_3^-$  before light limitation could be attained. Accordingly, the maximum steady-state biomass levels at all pH levels tended to be higher in the freshwater cultures (Figs. 2 and 3). This made it impossible to compare the effects of pH on productivity between the freshwater and marine species; but still, for the purpose of the study, the relative shapes of the pH-biomass curves in Figs. 2 and 3 are useful for discerning the importance of pH control in algal mass culture operation.

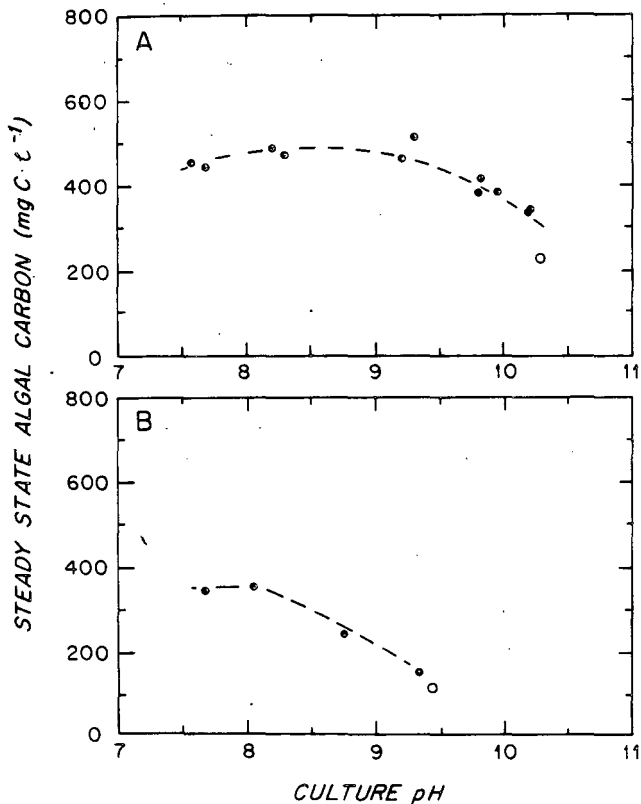


Fig. 2. Effect of culture pH on steady-state concentration of algal carbon for marine continuous culture maintained at a dilution rate of  $\approx 0.5 \cdot \text{day}^{-1}$ : A, *Phaeodactylum tricornerutum*; B, *Dunaliella tertiolecta*; ●, pH-stat control; ○, continuous air.

The highest pH levels attained were  $\approx 10.6$  for the freshwater species,  $\approx 10.3$  for *Phaeodactylum tricornerutum*, and  $\approx 9.4$  for *Dunaliella tertiolecta* (Table I). In all

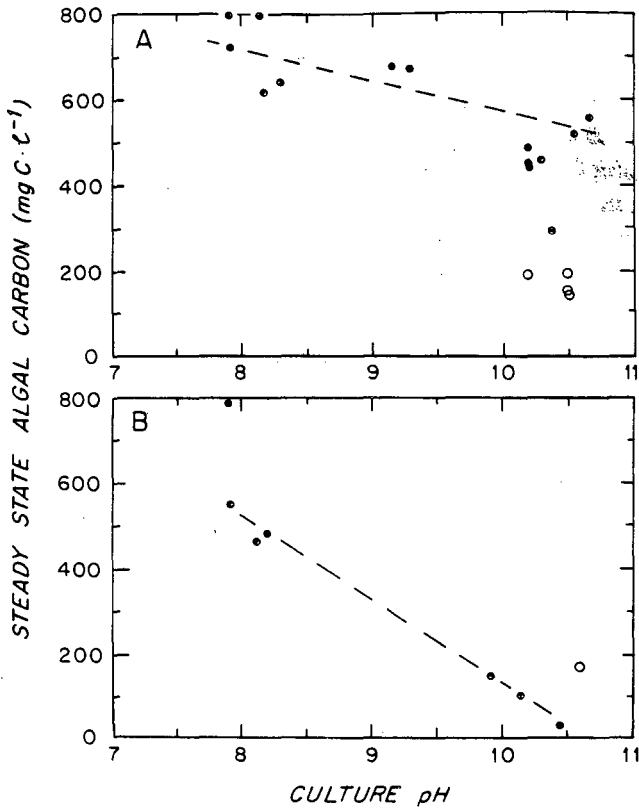


Fig. 3. Effect of culture pH on steady-state concentration of algal carbon for freshwater continuous cultures maintained at a dilution rate of  $\approx 0.5 \cdot \text{day}^{-1}$ : A, *Scenedesmus obliquus*; B, *Chlorella vulgaris*; ●, pH-stat control; ○, continuous air.

cases the pH maxima were the same regardless of whether the pH was regulated by the pH-stat system or allowed to increase via continuous bubbling of air without external control, although biomass production at the pH maxima was proportional to mass influx of inorganic carbon (Figs. 2 and 3). Although data on pH responses by marine algae are limited, numerous marine species appear to be unable to tolerate pH values much above 9.5 (Humphrey, 1975; Goldman, 1976), and typically grow optimally in a narrow pH range bracketing the pH of sea water which is  $\approx 8.1$  to 8.3 (Kain & Fogg, 1958a,b, 1960; Hayward, 1968; Humphrey, 1975). Yet, a few marine species, particularly *Phaeodactylum tricornerutum*, seem to behave like many freshwater algae and are capable of growing at pH levels up to and above 10 (Hayward, 1968; Humphrey, 1975; Goldman, 1976), even though

their pH optima are closer to 8 (Kain & Fogg, 1958a,b, 1960; Hayward, 1968).

There are no clearcut reasons why freshwater chlorophytes should be capable of tolerating a much wider range of pH than marine species. Intuitively, it would seem that freshwater species have acquired a tolerance to both acidic and alkaline pH as an adaptative response to widely fluctuating pH levels that occur commonly in many productive freshwater environments (Goldman *et al.*, 1972). Brock (1973), in fact, has suggested that freshwater eukaryotic algae probably evolved from acidic habitats where they could grow free from competition with blue-green algae; the latter algae have little tolerance to pH values  $<4$ , and do not, in general, grow well even in mildly acidic environments (Brock, 1973). In contrast, most natural marine algae probably never have evolved any physiological potential for thriving in extreme pH environments because the pH of sea water is so well regulated. Both *Dunaliella tertiolecta* and *Phaeodactylum tricorutum* are, however, tide-pool algae and, thus, would be expected to have evolved the ability to tolerate widely fluctuating environmental conditions. Yet, for reasons unknown, only the latter of the two species has become adapted to alkaline conditions.

With the exception of the results with *Dunaliella tertiolecta*, the observed limitations on growth at high pH seem to be due to metabolic inhibition. The two species that were least affected by changing pH, *Scenedesmus obliquus* (Fig. 3A) and *Phaeodactylum tricorutum* (Fig. 2A), displayed a threshold response when grown under pH-stat control and when the respective pH maxima were exceeded. Up to these pH maxima steady-state biomass levels were only moderately affected by pH, but once these pH values were exceeded, steady state could not be sustained and cell washout resulted.

The threshold response in the air-grown cultures of *Scenedesmus obliquus* provides a clue as to the nature of the inhibition. In this case, once steady state was reached, we measured a significant daily oscillation in pH around the threshold value of 10.6 that ranged from 9.4 to 11.2 in one experiment and 10.1 to 10.9 in another (Fig. 4). This oscillation may have been caused by a metabolic feedback mechanism that was manifested as a decrease in  $\mu$  below  $0.5 \cdot \text{day}^{-1}$  (the steady-state dilution rate) when the threshold pH was exceeded, leading to cell washout and a concomitant decrease in pH. When the pH dropped below an inhibitory level of  $\approx 10.6$ , the restraint on  $\mu$  was lifted so that both cell biomass and pH increased to complete the cycle. The rather large amplitude in this cycle ( $\approx 1-1.5$  pH units), which was not observed with the other species, may reflect a relatively sluggish feedback mechanism.

Chemical alterations in the growth media may also have been a major determinant of how each species responded to high pH. This is particularly true of *Dunaliella tertiolecta* and many other marine algae, which do not grow at pH  $>9.3$ . Due to the presence of large amounts of bivalent cations such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in sea water, the availability of essential, but sparingly soluble nutrients for marine algal growth such as phosphorus and trace metals is far more dependent on pH

than in comparable freshwater growth systems. Hence, the unique ability of *Phaeodactylum tricornutum* to tolerate pH values  $>9.3$  simply may reflect a reduced requirement for an essential nutrient that becomes less available with increasing pH.

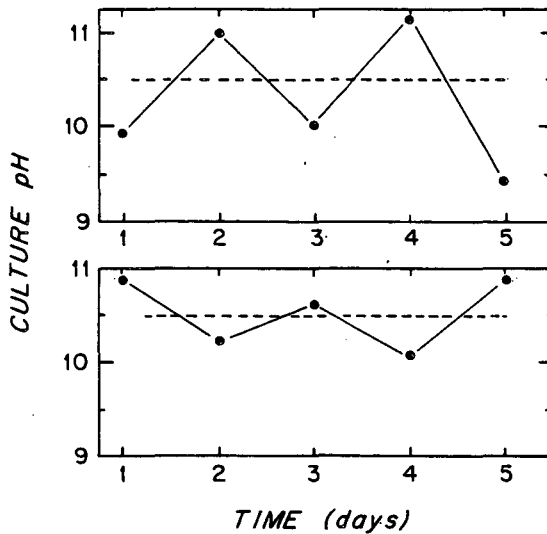


Fig. 4. pH oscillation around steady-state pH of  $\approx 10.6$  for cultures of *Scenedesmus obliquus* grown with continuous air supply at dilution rate of  $\approx 0.5 \cdot \text{day}^{-1}$ .

Alternatively, *P. tricornutum*, like freshwater blue-green algae (Murphy *et al.*, 1976), may excrete chelators that sustain the availability of trace nutrients at high pH (see Armstrong & Boalch, 1960). Such considerations were beyond the objectives of the current research, but serve to demonstrate the tremendous difficulties in discerning cause-effect relationships between algal growth and pH, particularly in marine systems. Our inability to culture both marine species in growth medium containing  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  at levels comparable to that in the freshwater medium adds still another dimension to the complex relationships between aqueous chemical processes and algal growth.

#### pH CONTROL OF BIOMASS AND CELLULAR CONSTITUENTS

All the test species grew best at low pH; maximum steady-state biomass was attained at pH 8.0–8.2 with the marine algae (Fig. 2) and at pH 7.9 with the freshwater species (Fig. 3). When the pH, however, was increased into the alkaline region, we observed two distinctly different types of response. On the one hand, both *Phaeodactylum tricornutum* (Fig. 2A) and *Scenedesmus obliquus* (Fig. 3A) were relatively unaffected by varying pH, and biomass levels for both species varied by no more than  $\approx 30\%$  over the range of pH tested. On the other hand,

*Dunaliella tertiolecta* (Fig. 2B) and *Chlorella vulgaris* (Fig. 3B) were extremely sensitive to alkaline pH so that biomass levels at the upper pH limits were only a small fraction of the maximum values attained at lower pH.

On the basis of the above results, it is intuitively obvious that *Phaeodactylum tricoratum* should be successful in intensive marine cultures that are poorly buffered. Clearly, once the pH rises  $> \approx 9.3$  *P. tricoratum* can grow without competition from species such as *Dunaliella tertiolecta* because of the latter's inability to tolerate high pH. Pruder & Bolton (1979) found the same sensitivity to high pH in *Thalassiosira pseudonana* (3H). Thus, the repeated dominance of *Phaeodactylum tricoratum* in large-scale outdoor cultures may be linked to poor pH control. As we shall, however, demonstrate in Part II of this study (Goldman

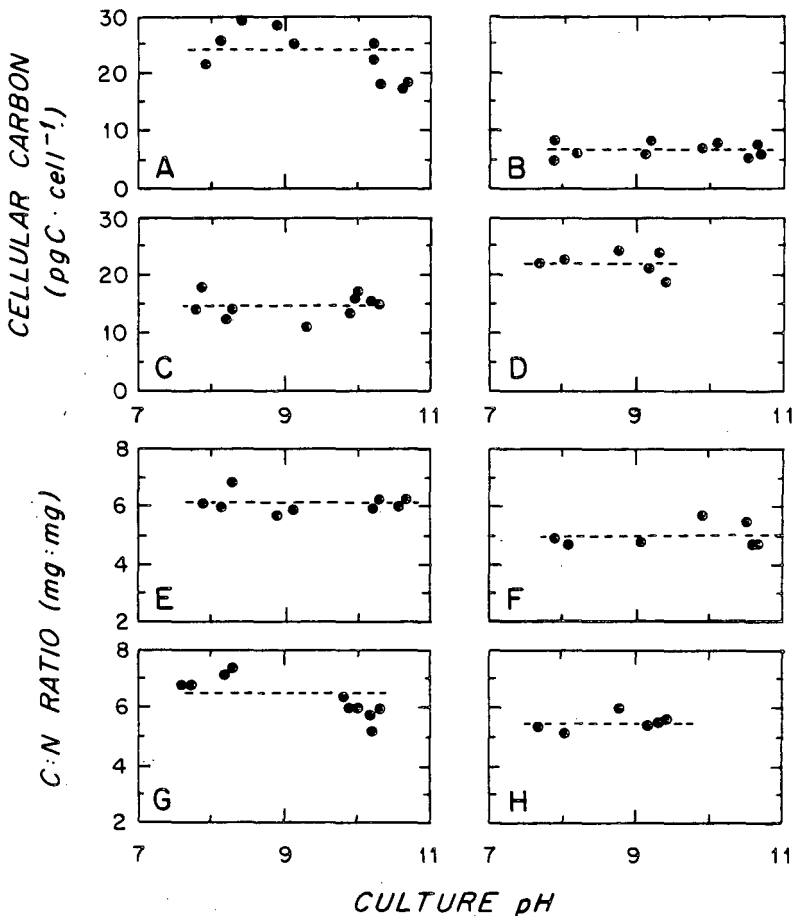


Fig. 5. Variations in steady-state cellular constituents of freshwater and marine algae with culture pH: A-D, cellular carbon; E-H, cellular nitrogen; A,E, *Scenedesmus obliquus*; B,F, *Chlorella vulgaris*; C,G, *Phaeodactylum tricoratum*; D,H, *Dunaliella tertiolecta*.

*et al.*, 1982), pH control alone is not the solution to maintaining other more desired species in culture.

Even though the four test species responded differently to high pH, the cellular constituents (cell quotas) of carbon and nitrogen for each organism remained constant at all levels of pH (Fig. 5 and Table I). The magnitude of these cell quotas, together with the constant and low cellular carbon:nitrogen ratios of 5.0 to 6.5 (by wt), are indicative of well-nourished cells and non-nutrient limiting conditions (Goldman, 1980). Therefore, variations in pH seem to have little effect on the distribution of macromolecular components in algal cells, although such changes result in gross alterations in membrane transport processes and metabolic functions involved in intracellular pH regulation (Smith & Raven, 1979; Raven, 1980).

#### pH CONTROL IN MASS CULTURES

For mass culture operation the necessity for pH control will be determined by the particular application. For situations in which the maintenance of specific species is not necessary and optimization of algal biomass production is the major objective, such as in waste-water treatment or energy production applications (Goldman, 1979), pH control probably is not required because for some species productivity is affected only slightly by high pH (Figs. 2A and 3A). For those mass culture applications that require the maintenance of specific species, such as aquaculture (Goldman & Stanley, 1974) or the production of chemical derivatives (Dubinsky *et al.*, 1978), pH control may be necessary. Economic considerations will dictate the choice of such a technique.

#### ACKNOWLEDGEMENTS

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