BIOENGINEERING ASPECTS OF INORGANIC CARBON SUPPLY
TO MASS ALGAL CULTURES

Final Report

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A. INTRODUCTION

As has been widely stated, there is potential for using algal mass cultures to solve either singularly or in combination numerous environment problems (Goldman, 1979a). Traditionally, the main interest in algal mass cultures has been for single-cell protein (SCP) production and wastewater treatment; but recently interest in extracting useful energy from algae through methane production has been advanced (Benemann et al, 1977a; Hall, 1979). This approach has serious limitations, however, because there is an upper limit to photosynthetic efficiencies that may prevent attainment of a favorable net energy balance with algal mass cultures (Goldman and Ryther, 1977). A more promising new application for algal mass cultures is the production of various biochemical extracts from algal biomass. Through proper selection of algal species and culture growth conditions, it may be possible to alter the chemical composition of algae so that production of desired algal derivatives such as lipids, particularly hydrocarbons, is favored. To this end, considerable research currently is in progress.

Regardless of the application, the basic biotechnology of large-scale outdoor cultures involves many common features, particularly in the requirement for adequate nutrients such as carbon, nitrogen, and phosphorus to ensure that light is the sole limiting yield determinant. Whereas the required quantities of nitrogen and phosphorus are fairly simple to estimate, those for inorganic carbon are far more complex (Goldman, 1979c). Even though the actual quantities of organic carbon

produced via photosynthesis can be calculated in the same manner as for nitrogen and phosphorus, the total amount of inorganic carbon required is much more difficult to calculate. This is because inorganic carbon is distributed among the chemical species CO_2 (aqueous), H_2CO_3 , HCO_3^- and CO_3^{2-} in an exceedingly complex chemical equilibrium system which is controlled by two parameters, alkalinity and pH. In natural fresh and marine waters this chemical system constitutes the main buffering system; losses of inorganic carbon through photosynthesis result in the destruction of buffering capacity, leading to a rise in pH, which can adversely affect algal growth in a number of ways (Goldman 1973).

Normally, the transport of CO_2 from the atmosphere cannot keep pace with algal assimilation of CO_2 during intense algal growth and a rise in pH to over 10 is not uncommon in eutrophic natural waters and mass culture systems (Goldman et al., 1972). Thus, in algal mass cultures to avoid the combined problems of inorganic carbon limitation and pH rise, inorganic carbon as CO_2 -enriched air is usually supplied via some aeration scheme or by creating sufficient turbulence so that sufficient CO_2 can be transferred from the atmosphere. Mixing can, to some degree, enhance CO_2 transport from the atmosphere; but, because of the very low concentration of CO_2 in the atmosphere (0.03 percent), the transport gradient is always small and CO_2 mass transfer is ineffective unless very turbulent mixing is employed. However, mixing is required in algal mass cultures for several other reasons: to prevent settling and subsequent decay of organic matter, to prevent thermal stratification, to break down diffusion gradients of essential nutrients which could develop at the cell surface

in very intense mass cultures, and most important, to provide uniform cell exposure to light because self-shading of cells exists in thick cultures.

The technology and resulting economics of providing adequate CO₂ and mixing in algal mass cultures is amazingly undeveloped considering the substantial research effort now underway to mass culture various freshwater and marine algae for bioconversion applications (Goldman, 1979c). The problem of delineating the requirements for carbon dioxide is exceedingly difficult and the major questions still to be addressed are the relative importance of mixing (for solving both carbon-limitation and non-carbon related problems), pH control, and quantity and source of inorganic carbon necessary for maximizing algal yields. Therefore, a major objective of this research program has been to address the question of inorganic carbon supply to algal mass cultures primarily from a scientific basis. With a firm understanding of the chemical-biological interactions involved in carbon availability, rational decisions can be made regarding the engineering design of carbon dioxide supply systems for large-scale cultivation systems.

B. LITERATURE REVIEW

As shown previously (Goldman et al., 1972), the interrelationships between inorganic carbon aqueous chemistry and algal growth are exceedingly complex. Difficulties in sorting out the effects of carbon supply and pH on algal growth are well-established. Yet an understanding of these effects is crucial to developing the conditions allowing for yield optimization.

1. pH Effects on Algae

Hydrogen ion concentration exerts a profound effect on algal growth and metabolism, largely through its effect in the protonation and deprotonation of enzyme systems. Algae of various species show different pH optima for growth, and it is plausible that these are due to the effect of H^+ from the bulk fluid. The cell surface pH is thus lower than in the bulk fluid, causing the observed pH optima for surface enzyme reactions to be shifted to the alkaline region, as compared to the lower observed pH optima for the same enzyme reaction in solution (McLaren and Packer, 1970; Katchalski et al., 1971). The lowered pH at the algal cell surface undoubtedly results in an increase in the $CO_2(aq)$ concentration in this region over that observed in the bulk fluid. Because cell surface pH is a function of cell surface isoelectric point - distinct for each algal species - it is possible that different algal species, though seeming to have different pH optima for growth, may in fact, at these observed pH optima values, have closer surface pH values and resulting inorganic carbon species distribution. No studies have been attempted to examine the effect of cell surface pH on algal growth, although Kolin (1955) and Ives (1959) studied the electrical properties of several algae while investigating method for their removal from the liquid phase.

The optimum pH for several enzymes in the Calvin cycle was studied by Preiss et al. (1967) and Bassham et al. (1968). Preiss et al. (1967) found that by increasing the magnesium concentration the pH optimum for fructose diphosphatase activity, an enzyme of the Calvin cycle, was decreased. Bassham et al. (1968) found similar effects of magnesium on the pH optimum of ribulose diphosphate carboxylase.

Ouellet and Bensen (1952) showed that the initial incorporation of CO_2 in Scenedesmus switched from three-carbon compounds to four-carbon compounds when the pH was raised from the acid to the alkaline regions. pH control of enzyme activity was felt to be the main factor controlling the shift in carbon compound synthesis.

King (1970) suggested that pH, through its control of the free CO_2 concentration, played a major role in regulating the distribution of algal species in natural waters. Although this hypothesis has never been thoroughly examined, pH may play a very important role in the selection of certain algal species in natural waters for other reasons, presently unknown. McLachlan and Gorham (1962), Jackson (1964), Holm-Hansen (1967), Brock (1973), and Shapiro (1973) pointed out that blue-green algae appear to be favored by a more alkaline environment in natural fresh-waters. Brock (1973), in fact, suggested that blue-green algae cannot tolerate very low pH values (<5), whereas numerous eukaryotic algae can flourish in such environments. Allen (1935) demonstrated species change in oxidation ponds with increases in pH from the neutral to the alkaline regions. Keenan (1975) claimed that pH effects not related to inorganic carbon species distribution were the main regulators of photosynthetic rates in the blue green alga Anabaena flos-aquae. Photosynthetic rate was inversely proportional to pH in the range 6.3 - 10.3.

Zabat (1970) grew Chlorella pyrenoidosa (high temperature strain) in a phosphorus-limited continuous culture, and showed a decrease in cell yield with increasing pH values between 7.0 and 8.25. Emerson and Green

(1938), on the other hand, could show no change in Chlorella photosynthetic rates over a pH range from 4.6 to 8.9.

Soltero and Lee (1967), in demonstrating an automatic pH control device for algal cultures, gave evidence that optimum growth of Scenedesmus occurred at a pH of 7. This was in contrast to the work of Witt and Borchardt (1960) and Gates and Borchardt (1964) who could show little change in the growth of Scenedesmus over a wide pH range, although best growth was observed at a pH of 8.3. Brown (1969), when growing Scenedesmus on agricultural tile drainage (alkalinity = 350 mg/l as CaCO_3) in which pH was controlled with "Good" organic acid buffers achieved best growth at a pH of 8.4 (as compared to other tested pH values of 6.15, 7.5, and 10.5), in agreement with the results of Witt, Gates and Borchardt.

Gerloff et al. (1952) showed that the maximum yield of the blue-green alga, Microcystis aeruginosa, cultured in an unbuffered medium, occurred at a pH of 10. Rand and Nemerow (1965) presented similar results for the same species, while McLachlan and Gorham (1961) and McLachlan (1962) observed little change in growth in a pure culture over a pH range of 6.5 to 10 in a well-buffered medium. When they tried to grow this alga together with Scenedesmus at a pH of 7.4 they obtained less than one third of the growth reached by the blue-green alga in pure culture. There appeared to be a definite competitive effect at the lower pH, indicating that only at the higher pH values would the blue-green alga predominate. Eberly (1967) reported that in batch cultures of Oscillatoria agardhii, another blue-green alga, those cultures with the highest initial pH values (up to 10) reached the exponential phase earliest, but that all cultures eventually reached the same level of maximum biomass.

More recently, I (Goldman et al., 1974) demonstrated that under inorganic carbon limitation growth rates of two freshwater green algae were controlled by the total inorganic carbon concentration and that even within a small range of pH between 7.1-7.6 half-saturation coefficients for growth increased with increasing pH. Gavis and Ferguson (1975) expanded on this concept by developing a model to account for any mass transport (or diffusion) limitations that might exist at high pH when CO_2 concentrations are very low and CO_2 is the only form of inorganic carbon available. In my study (Goldman et al., 1974) no attempt was made to determine which form of inorganic carbon was utilized since the imposed growth conditions were such that the rate of CO_2 provided from HCO_3^- via chemical reactions was always greater than the rate at which inorganic carbon was assimilated by the test algae. Hence, the effect of total inorganic carbon on growth rates was indistinguishable from any of the inorganic carbon species. This situation more than likely would not occur in mass cultures if HCO_3^- was the only source of inorganic carbon. In addition, unless good mixing is established the mass transfer of CO_2 to an algal cell could become limiting, as suggested by Gavis and Ferguson (1975).

Moss (1973) found that freshwater species common to oligotrophic waters could not tolerate culture pH values much above 8.6, whereas those algae found in eutrophic environments thrived when the pH was over 9.3. Talling (1976) observed that certain freshwater diatoms could actively grow in pH environments >10.5 . In this regard, I have observed that when the green algae Scenedesmus quadricauda and Selenastrum capricornutum were grown in HCO_3^- -buffered continuous cultures with no other carbon source,

growth of these species, and concomitant destruction of the buffer system, led to pH values of 11.2 and 10.6 respectively (Goldman, unpublished data).

One interesting and very important blue-green alga for mass culture applications in the filamentous species Spirulina (Clement 1975). This species is found in abundance in highly alkaline water bodies such as the African Rift Lakes and appears to prefer both a high pH and HCO_3^- environment. Kosaric et al. (1974) found a pH optimum of 9.5 for this species, but no attempt was made to separate out carbon limitation effects from those solely due to pH. Benemann et al. (1977b) likewise showed a pH optimum of 9-10 for Spirulina and further demonstrated that HCO_3^- could be replaced by bubbled CO_2 without any effects on growth rates as long as the pH was controlled between 9-10.

Surprisingly little information is available on pH effects on marine microalgae. Seawater is quite strongly buffered (2 meq HCO_3^-) and the pH is typically ~8.1 (Skirrow, 1975), so that in natural waters algae rarely experience pH variations.

Paasche (1964) and Swift and Taylor (1966) claimed to isolate the effects of pH on the growth of marine coccolithophorids. Paasche (1964) showed that carbon uptake in the coccolithophorid Coccolithus huxleyi was highest at a pH of 7.5. Swift and Taylor (1966) showed an optimum pH of 7.8 for cell division of the coccolithophorid Cricosphaera elongata.

Yet, in previous mass culture work with marine microalgae grown on seawater-wastewater mixtures, pH values >10 were observed during intense

growth (Goldman and Ryther, 1975; Goldman et al., 1975) In these experiments certain diatoms such as Phaeodactylum tricornutum, Amphiprora, Amphora and Nitzschia closterium always dominated. In similarly controlled laboratory experiments with monocultures of species such as the chlorophyte Dunaliella tertiolecta and the diatom Thalassiosira pseudonana (3H) the pH never rose above 9.5, resulting in corresponding lower biomass levels (Goldman, 1976). More recently (Goldman, 1979c), I confirmed that P. tricornutum could tolerate and grow well at high pH (>9.4), whereas the diatoms Skeletonema costatum, and Thalassiosira pseudonana (13-1), the chrysophyte Monochrysis lutheri and D. tertiolecta did not grow well at pH values approaching 9 when HCO_3^- was the sole carbon and buffer source. Pruder and Bolton (1979) likewise found that growth of T. pseudonana (3H) was severely curtailed when the pH reached 9.

These results provide circumstantial, but strong evidence that pH may be an important factor influencing species competition in outdoor mass cultures. Whether purely pH effects on cellular metabolism or indirect effects of pH on the availability of specific inorganic carbon species is the cause of the above results cannot be answered from the data available.

2. Sources of Inorganic Carbon for Algal Growth

The question of whether aqueous CO_2 or HCO_3^- is the actual substrate for algal growth has been of long standing interest to plant physiologists and phycologists (Raven, 1970). However, for the most part, difficulties in separating out pH effects on cell physiology and culture medium chemistry from those effects on the equilibrium distribution of the chemical species, CO_2 , H_2CO_3 , HCO_3^- , and CO_3^{2-} and on the rate reactions in this

chemical system have made it extremely difficult to interpret the vast body of literature on this subject (Goldman, 1973).

The main difficulty stems from the fact that when bicarbonate alkalinity is present in a culture medium it is usually impossible to distinguish between HCO_3^- and CO_2 assimilation because typically the conversion of HCO_3^- to CO_2 via the following reactions:

is fast enough relative to the demand for inorganic carbon by algae so that a rate bottleneck on the inorganic chemical side does not exist. Algal uptake of inorganic carbon via reactions (1) (dominant at $\text{pH} < 8$) or (2) (dominant at $\text{pH} > 10$) leads to a pH rise, but no change in alkalinity (Goldman et al., 1972). At the same time the proportion of HCO_3^- to CO_2 increases dramatically. In many previous studies the simultaneous increase in pH and the ratio $\text{HCO}_3^-/\text{CO}_2$, together with good growth at high pH has led to the conclusion that HCO_3^- was the substrate (Raven, 1970). Unfortunately, this technique by itself, does not provide conclusive evidence for direct bicarbonate use because of possible species dependent pH effects on cell physiology and because of the rate bottleneck question discussed previously.

Recently Talling (1976) and Miller and Colman (1980) have suggested that direct bicarbonate use is common at high pH by claiming that rates

of photosynthetic carbon assimilation in their experiments exceeded the rate of CO_2 production from HCO_3^- via reactions (1) and (2). In both cases actual carbon assimilation was measured indirectly (i.e. pH changes or O_2 evolution) so that it is difficult to assess their results. Lehman (1978) in an elegant study used rapid ^{14}C -pulse labelling according to the procedure established by Cooper and Filmer (1969) to show that both HCO_3^- and CO_2 are transported across the cell membrane to the chloroplast in the green alga Chlamydomonas reinhardtii. Sikes et al., (In press), using the same technique, found that CO_2 was the substrate for photosynthesis in the marine coccolithophore Coccolithus huxleyi, but that HCO_3^- was used directly in coccolith formation.

Facilitated transport of HCO_3^- along with CO_2 assimilation to provide an adequate supply of CO_2 at the sites of photosynthetic carbon assimilation via the Calvin cycle appears to be a common mechanism for ensuring an adequate supply of CO_2 when total inorganic carbon is in limited supply. Physical models of such processes have been demonstrated both in liquid and artificial membrane-liquid phases (Longmuir et al., 1966; Enns, 1967; Ward and Robb, 1967; Broun et al., 1970). A critical component of these facilitated transport mechanisms is the requirement for the enzyme carbonic anhydrase, which catalyzes reactions (1) and (2) in both directions.

Carbonic anhydrase commonly is present in algal protoplasm (Litchfield and Hood, 1964). Nelson et al., (1969), Graham and Reed (1971), Graham et al., (1971); Findenegg (1974) and Berry et al. (1976) have demonstrated that the enzyme is found in far greater quantities in algal cells grown

on air than those on 1-5 percent CO_2 enriched air, suggesting a role for this enzyme under carbon-limiting situations. Shiraiwa and Miyachi (1978) and Miyachi and Shiraiwa (1979) found that CO_2 was the substrate for chloroplasts of both spinach and the green alga Byropsis maxima and whole Chlorella vulgaris cells by observing an enhancement of photosynthesis when carbonic anhydrase was added to a medium containing HCO_3^- and a suppression when the enzyme was added to medium through which air or CO_2 -enriched air was bubbled. In the former case the enzyme catalyzed the forward reactions (1) and (2) making more CO_2 available for growth, and in the latter case the backward reactions (1) and (2) were catalyzed so that free CO_2 was removed, leading to a reduction in photosynthesis. Findenegg (1976) showed that Scenedesmus cells conditioned on 2 percent CO_2 -air and then switched to air (0.3 percent CO_2) at first did not contain carbonic anhydrase and could not utilize HCO_3^- . The K_s (half saturation coefficient) for photosynthesis was $\sim 100 \mu\text{M CO}_2$. After 1-3 hrs of exposure to air the cells readily used HCO_3^- , carbonic anhydrase was present, but the K_s value was still $\sim 100 \mu\text{M CO}_2$. Finally, after >4 hrs of aeration with air, carbonic anhydrase remained present, but the K_s value decreased to $\sim 5 \mu\text{M CO}_2$. Berry et al., (1976) demonstrated the same low affinity ($K_s \approx 25 \mu\text{M CO}_2$) for Chlamydomonas cells grown on 5 percent CO_2 -air as opposed to high affinity ($K_s \leq 2.7 \mu\text{M CO}_2$ for air-grown cells. The maximum rates of photosynthesis and growth rates were virtually identical with either CO_2 source. The increased efficiency of low- CO_2 adapted cells was eliminated by addition of Diamox, an inhibitor of carbonic anhydrase activity. Badger et al., (1977, 1978) demonstrated that there was an internal pool

of CO_2 present in both Chlamydomonas and the blue green alga Anabaena that increased as a function of degree of external CO_2 limitation. They suggested that a HCO_3^- influx pump existed in this species which requires ATP expenditure.

3. Inorganic Carbon Supply for Maximum Photosynthesis

There appears to be general agreement that maximum photosynthetic rates of species such as Chlorella and Scenedesmus can be sustained on CO_2 concentrations no greater than that present in atmospheric air (~0.035 percent). For example, in most of the experiments dealing with carbon effects on Chlorella it was found that a CO_2 concentration of from 0.01 to 0.03 percent was adequate for maintaining the maximum rate of photosynthesis (Emerson and Green, 1938; Briggs and Whittingham, 1952; Steemann Nielsen, 1953, 1955a). Similar results have been observed for the effect of carbon dioxide concentration on growth rates in a wide variety of freshwater and marine algae (Swift and Taylor, 1966; Ingle and Colman, 1975; Berry et al., 1976; Small et al., 1977; Pruder and Bolton, 1979). In all the above cases growth rates were maximum at CO_2 concentrations in bubbled gas of ~0.03 percent. However, it should be noted that the percent CO_2 in air is a relatively meaningless term in trying to describe the amount of CO_2 required for maximum photosynthesis in culture work if no accounting is made for the concentration of CO_2 in solution which is really available to the algae. The amount really available is a function of the sparging rate and its effect on the CO_2 tension at the cell surface where the demand for inorganic carbon occurs.

Markl (1977), in an eloquent study with continuously cultured Chlor-
ella, actual was able to estimate the concentration of CO_2 at the cell
surface corresponding to the half saturation coefficient with respect to
photosynthetic rate. For a 4-fold variation in light intensity he found
 K_s values to be $<3 \text{ ppm } \text{CO}_2$ (<0.0003 percent). This important observation
demonstrates that algal affinity for CO_2 is tremendously high and that
the actual mass transport of CO_2 from the gas phase to the bulk fluid and
then onto the cell surface plays a critical role in establishing whether
or not a carbon limitation is present. Clearly, the presence of bicarbon-
ate alkalinity helps to alleviate this potential mass transport bottleneck
by providing a supply of CO_2 via dehydration at the cell surface or by
facilitated transport of the ion itself across the cellular membrane. The
presence of carbonic anhydrase when CO_2 is in limited supply (~ 0.035 per-
cent CO_2 -air) suggests that algae have the ability to maintain maximum
transport rates of inorganic carbon across the cell membrane so that the
rate limiting step for maximum photosynthesis is the initial carboxylation
reaction of the Calvin cycle involving the enzyme ribulose 1-5 bisphos-
phate carboxylase (RUBP Case). The critical factor then controlling
photosynthesis in a dense culture is the mass flux of inorganic carbon
from the gas phase to the cell surface, which, in turn, is regulated by
culture geometry, liquid mixing, aeration rate, bubble size, and the par-
tial pressure of CO_2 in the aerating gas. Bicarbonate alkalinity, through
its influence on pH and in acting as a reservoir for inorganic carbon for
photosynthesis, similarly plays an important role in the overall kinetics
of inorganic carbon uptake by algae.

4. Inorganic Carbon Supply to Large Scale Cultures

It has long been recognized that gaseous CO_2 must be supplied to algal mass cultures to optimize yields. Cook (1951) is one of the earliest mass culture experiments with Chlorella suggested that a 5 percent CO_2 mixture with air bubbled into the culture optimized inorganic carbon requirements. The early Japanese workers, developed with the "outdoor bubbling technique" (Morimura et al., 1955) and the "open circulation method" (Kanazawa et al., 1958) for optimizing the supply of CO_2 to algal cultures, but found that, because the culture surface was exposed to the atmosphere, "an enormously large quantity of CO_2 -enriched air is required for aeration, and a considerable part of the CO_2 is wasted without being utilized by algal cells". Similarly, Oswald and co-workers at Berkeley, in their early experiments with wastewater-grown freshwater algae, found substantial inorganic carbon limitations existing in their cultures that could be overcome by enrichment with CO_2 -air mixtures bubbled into the cultures (Ludwig et al., 1951, Oswald et al., 1953). In the German mass culture experiments at Dortmund, Germany (Soeder, 1976) only about one-half the CO_2 supplied through aeration was actually assimilated by the algae. In none of these studies was any attempt made to optimize the efficiency of CO_2 use by consideration of the interrelationships between the CO_2 - HCO_3^- - CO_3^{2-} chemical equilibrium system controlled by the alkalinity present and the added gaseous CO_2 as they were affected by inorganic carbon assimilation during photosynthesis.

Shelef (1976) found that adding inorganic carbon either as bubbled pure CO_2 or as HCO_3^- to raw sewage already high in alkalinity (9.6 mM C/l) had virtually no effect on increasing yields of freshwater green algae. pH values varied from 8.4 to the control and HCO_3^- ponds to 7.8 in the CO_2 ponds, indicating that the natural alkalinity in this case provided the necessary carbon. These results suggest that HCO_3^- additions to waters typically lower in alkalinity than in the above situation may be an efficient technique to both control pH and supply the required carbon.

Pipes (1962), in a laboratory study showed for inorganic-carbon limited growth of algae in continuous culture, that there was a linear relationship between steady state algal concentration and cell residence period for a fixed rate of CO_2 -air addition. This was the first attempt to optimize the addition of CO_2 to a mass algal culture. Unfortunately however, no consideration was given to the role of alkalinity in regulating the availability of the CO_2 .

Some attempts to add very high CO_2 in air mixtures have resulted in apparent toxicity effects (Steemann-Neilsen, 1955, Sorokin, 1962, Brown, 1971, Shelef, 1976). Others (Tew et al., 1962, Fowler, et al., 1972) have demonstrated no adverse effects on Chlorella using virtually 100 percent CO_2 additions. An important factor not considered in any of these studies is the rate of addition and the efficiency of diffusion.

Work at the Trebon mass culture laboratories of Czechoslovakia was addressed towards answering some of the fundamental questions of CO_2 mass transfer and diffusion of liquid cultures of growing algae (Necas and Lhotsky, 1966, 1967, 1968, 1969, 1970), and although significant

technological advances were made in these studies, once again no accounting was made for the effects of alkalinity and pH on the efficiency of CO_2 transfer and use. In some experiments 60 percent utilization efficiencies were achieved, however.

With marine systems the requirements for gaseous CO_2 are not as severe as with freshwater cultures because of the substantial alkalinity (two milliequivalents/l) and resulting total inorganic carbon concentration (24 mg/l) present in seawater. Yet, even in these systems inorganic carbon-limitation can exist and high culture pH levels can occur if supplementary CO_2 is not provided. Goldman and Ryther (1975) showed that very strong mixing to enhance CO_2 transport from the atmosphere was required in mass cultures of marine diatoms grown on wastewater-seawater mixtures to meet the full inorganic carbon requirements of the algae and prevent the pH from rising to growth-inhibiting levels; even still, mid-day pH values rose to over 10 as CO_2 derived from HCO_3^- present was utilized along with the CO_2 added during mixing. No attempt was made to add CO_2 -enriched air and control the pH, however.

Interpretation of the divergent results reviewed in this section is almost impossible. Although an optimum pH for algal growth has been demonstrated in a number of studies, few have been able to show that pH was the only factor affecting the observed growth. The difficulty in separating pH effects on growth from those due to changes in the H_2CO_3 - HCO_3^- - CO_3^{2-} system, from the precipitation of other essential nutrients and from mixing effects has plagued many researchers.

In their recent cost analysis of large-scale biomass systems for bio-conversion of energy Ashare et al., (1978) investigated the requirements for CO₂ supply to a 100 square mile freshwater algal system. They considered both atmospheric air supply (0.03 percent CO₂) and power plant stack gas with a CO₂ content of ~10 percent as potential sources of inorganic carbon. They arrived at the startling conclusion that inorganic carbon supply via a network of pipes, headers and sparging units placed intermittently in the large-scale culture units would be prohibitively expensive even with enriched power plant exhaust gases. The analysis was based on a number of assumptions, many of which were criticized by Oswald and Benemann (1978).

The main point of the analyses (and the subsequent discussion of this analysis) is that very little information is available upon which to estimate rationally the carbon requirements of large-scale algal cultures and to design supply systems accordingly. For example, the analysis of Ashare et al., (1978) did not include consideration of the aqueous chemistry of inorganic carbon (i.e pH, alkalinity, and HCO₃⁻ concentration). Carbon requirements were treated as purely a gas transfer problem with no consideration given to the chemical reactivity of CO₂ with HCO₃⁻ and CO₃⁻², the fact that pH plays a crucial role in controlling the CO₂ gradient between gas-liquid phase, and that algal species differ considerably in their ability to tolerate pH variations and to utilize different inorganic carbon sources. Moreover, if CO₂ is supplied via gas transfer systems then the conditions for maximizing the efficiency of CO₂ transfer and assimilation by algae are not necessarily the same as those necessary to maximize yields from the standpoint of forcing available sunlight to be the sole limiting growth factor.

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C. INORGANIC CARBON SOURCES FOR ALGAL GROWTH

To determine the most efficient and economical method for adding dissolved inorganic carbon to mass algal cultures, in the current study HCO_3^- alkalinity and bubbled gas were compared as carbon sources. For the HCO_3^- study two sets of experiments were performed, one with the marine diatom Phaeodactylum tricornutum grown in artificial seawater medium, and the other with 3 freshwater green algae, Chlorella vulgaris, Scenedesmus obliquus, and Selenastrum capricornutum. The bubbled gas experiments were carried out primarily with S. obliquus, though, in several experiments the other two freshwater species were used.

1. Theoretical Considerations

The kinetics of inorganic carbon assimilation in a continuous culture are distinctly different depending on whether the carbon source is part of the influent liquid medium, as would be the case for HCO_3^- alkalinity, or it is supplied at a rate independent of the liquid medium flow rate, as for bubbled CO_2 . In both cases a mass balance for carbon can be established.

a. HCO_3^- Alkalinity: A critical consideration in developing a kinetic model of inorganic carbon uptake by microalgae when HCO_3^- alkalinity is the sole carbon source is that the rate reactions in the $\text{CO}_2 - \text{HCO}_3^- - \text{CO}_3^{2-}$ chemical system are not limiting. Then the uptake of a particular

carbon species should be indistinguishable from the entire inorganic carbon pool C_T [ML⁻³], defined as

$$C_T = CO_2 - C + HCO_3^- - C + CO_3^{2-} - C \quad (3)$$

in which $CO_2 - C$, $HCO_3^- - C + CO_3^{2-} - C$ are the concentrations of inorganic carbon [ML⁻³] in the respective chemical species. The relative fractions of the chemical species are a function of pH.

Considerable controversy exists as to whether microalgae can assimilate HCO_3^- directly along with CO_2 , or are obligate CO_2 users.⁷ For if a particular algal species can only assimilate CO_2 , than it is conceivable that reactions (1) and (2), which are relatively slow, could limit the rate of supply of CO_2 from the total inorganic carbon pool and place an overall limit on the rate of photosynthesis. The question is somewhat academic, however, for several well established reasons: 1) reactions (1) and (2) are still rapid enough to prevent a bottleneck in the chemical production of CO_2 relative to the demand of algae in most natural water situations,⁸ 2) facilitated transport of HCO_3^- across cell membranes along with CO_2 uptake to provide adequate CO_2 at the sites of photosynthesis within the cell seems to be a common characteristic of microalgae,^{9,10} and 3) production of the enzyme carbonic anhydrase, which catalyzes reaction (1) and (2), is enhanced when cells are grown at low CO_2 partial pressures.^{11,12}

In consideration of the above factors, the mass flux of inorganic carbon in a photosynthetic continuous culture in which HCO_3^- alkalinity is the sole carbon source is described as:

Change	Input	Output	Growth	
$V \frac{dC}{dt}$	$F C_{T_0}$	$- F C_{T_1}$	$- \mu C_A V$	(4)

in which dC/dt is the rate of change in inorganic carbon concentration $[\text{ML}^{-3}\text{T}^{-1}]$, V is the culture volume $[\text{L}^3]$, F is the liquid medium flow rate $[\text{L}^3\text{T}^{-1}]$, C_{T_0} and C_{T_1} respectively are the influent and effluent concentrations of total inorganic carbon $[\text{ML}^{-3}]$, C_A is the algal carbon concentration $[\text{ML}^{-3}]$, and μ is the specific growth rate $[\text{T}^{-1}]$. At steady state, and defining the dilution rate $D [\text{T}^{-1}]$ as F/V ,

$$\frac{dC}{dt} = 0 = D C_{T_0} - D C_{T_1} - \mu C_A \quad (5)$$

and,

$$C_A = D(C_{T_0} - C_{T_1})/\mu \quad (6)$$

However, at steady state $D = \mu$ and $C_{T_0} \gg C_{T_1}$ occurs when D is less than the washout rate ($= \hat{\mu}$, the maximum growth rate)⁸ so that

$$C_A \approx C_{T_0} \quad (7)$$

b. Bubbled CO_2 : When the source of inorganic carbon is bubbled CO_2 , a mass balance for the flux of inorganic carbon in a continuous culture is:

$$\begin{array}{cccc} \text{Change} & \text{In} & \text{Out} & \text{Growth} \\ V \frac{dC}{dt} & = R_0 - R_1 - \mu C_A V \end{array} \quad (8)$$

in which R_0 and R_1 respectively are the fluxes of inorganic carbon bubbled into and out of the culture $[\text{MT}^{-1}]$.

At steady state eq. (8) reduces to

$$C_A = (R_0 - R_1)/V\mu \quad (9)$$

However,

$$R = AG'P \quad (10)$$

in which A is a coefficient to incorporate temperature, atmospheric and water vapor pressure corrections to the molar volume of a gas and to convert from moles of carbon to mass, G' is the gas bubbling rate $[\text{L}^3\text{T}^{-1}]$, and P is the partial pressure of CO_2 in the bubbled gas. Then,

$$C_A = AG' (P_0 - P_1)/V\mu = AG (P_0 - P_1)/\mu \quad (11)$$

in which P_0 and P_1 are respectively the partial pressures of CO_2 in the influent and effluent gas, and G is the specific gas bubbling rate $[T^{-1}]$ ($= G'/V$).

An alternate way to define a mass balance with a bubbled gas is to consider the net gas transfer efficiency,¹³ so that

Change Net Gas Transport Growth

$$V \frac{dC}{dt} = K_L a (C_0 - C_1)V - \mu C_A V \quad (12)$$

in which C_0 and C_1 are respectively the dissolved CO_2 concentrations at saturation and in the culture filtrate, and $K_L a$ is the overall mass transfer coefficient $[T^{-1}]$.

Then, at steady state

$$C_A = K_L a (C_0 - C_1) / \mu = K_L a H (P_0 - P_1) / \mu \quad (13)$$

in which H is the solubility coefficient for CO_2 in water ($C = HP$).

By equating eqs. (11) and (13), it follows that

$$K_L a = (A/H) G = A' G \quad (14)$$

in which $A' = A/H$, a constant for a given set of environmental conditions.

2. Materials and Methods

a. Culture Methods: The continuous-culture apparatus (a bank of eight 0.5-liter cultures), the culturing protocols, and the experimental analyses were virtually identical to those described previously.^{14,15} Continuous lighting ($0.06 - 0.07 \text{ cal/cm}^2/\text{min}$), temperature control (20°C), and mixing with Teflon coated stirring bars were employed in all experiments. For those experiments in which HCO_3^- alkalinity was the sole source of inorganic carbon there was no gas phase and stirring was the only form of mixing employed. When bubbled gas was the carbon source it was introduced either as large bubbles ($\sim 1.3\text{--}1.9 \text{ cm dia.}$) through a port of the base of the culture, or as small bubbles ($\sim 0.2\text{--}0.4 \text{ cm dia.}$) through a glass-fitted sparger connected by a glass tube and positioned in the culture slightly above the stirring bar. Mixtures of CO_2 and air ranging from 0.036% CO_2 (air) to 100% CO_2 were obtained by blending the gases to a desired mixture in a 2-gas proportioner. The mixtures were then metered into the cultures through rotometers at flow rates ranging from 1 to 100 liters/day. In a few experiments helium was used in place of air in preparing the CO_2 mixtures. Liquid medium was metered into the cultures at a fixed dilution rate D of $\sim 0.5/\text{day}$ via a multichannel peristaltic pump (Harvard no. 1203). In one experiment, however, the % CO_2 level was held constant at 0.036% and D was varied. All medium tubing was glass except for a small section of silicone tubing inserted in the pump. Medium was kept in ice-filled styrofoam containers and changed daily from a larger supply stored in a refrigerator. A schematic view of one culture is shown in Figure 1.

b. Algal Cultures and Growth Medium: The freshwater chlorophytes Chlor-
ella vulgaris and Scenedesmus obliquus were obtained from the laboratory
of M. Gibbs at Brandeis University, the chlorophyte Selenastrum capricor-
nutum came from the U.S. Environmental Protection Agency in Corvallis,
Oregon, and the marine diatom Phaeodactylum tricornutum (clone TFX-1),
originally isolated from an outdoor mass culture,¹⁶ was available in the
authors' laboratory. The freshwater growth medium was a modification of
that used previously,⁸ and consisted of 2-15 mM NH_4Cl , 0.4 mM MgCl_2 ,
0.4 mM MgSO_4 , 0.2 mM CaCl_2 , 0.04 mM H_3BO_4 , and trace metals in a twofold
dilution of f medium.¹⁷ The artificial seawater medium was prepared
from 400 mM NaCl , 20 mM MgSO_4 , 20 mM MgCl_2 , 10 mM CaCl_2 , 10 mM KCl , 0.8
mM KBr , 0.2 mM H_3BO_4 , 0.1 mM Na_2SiO_3 , 0.06 mM NaH_2PO_4 , 0.45-0.9 mM NH_4Cl ,
and trace metals and vitamins as prescribed in f medium. For the HCO_3^-
alkalinity experiments a mixture of NaHCO_3 and NaCO_3 was added to give up
to 105 mg C/liter in the seawater medium and up to 190 mg C/liter in the
freshwater medium.

The buffer system used in the HCO_3^- experiments with seawater consis-
ted of up to 40 mM HEPPS, (N-2-hydroxyethylpiperazine-N'-3-propane-sulfon-
ic acid) resulting in a range of culture pH values between 7.8-9.3 depend-
ing on the concentrations of HCO_3^- and buffer added. The freshwater medium
was buffered with 25 mM phosphate, consisting of equi-molar concentrations
of K_2HPO_4 and KH_2PO_4 . In some freshwater experiments with the higher

HCO_3^- and CO_2 levels in bubbled gas up to 50 mM buffer was required to control the pH, and in other experiments no buffer was used. For the bubbled gas experiments culture pH values ranged between ~6.5 and 6.8 for $\% \text{CO}_2 \leq 0.17$, and decreased to ~6 when 1% or 100% CO_2 was used. When no buffer was added and with CO_2 levels of 0.036% the pH dropped to ~4.5. The pH levels for the HCO_3^- alkalinity experiments were more variable and generally increased from ~7 at low HCO_3^- alkalinity additions and 25 mM buffer to >9.5 when HCO_3^- alkalinity was high and no buffer was employed.

c. Chemical Analyses: Chemical analyses for C_{T_0} and C_{T_1} were carried out on a Dohrman DC-54 Ultra-Low Total Carbon Analyzer, modified for inorganic carbon analyses both on liquid and gas samples.¹⁸ The instrument has a precision of $\pm 10 \mu\text{g C/liter}$ (or $\pm 2\%$) and a detection limit of $\sim 50 \mu\text{g C/liter}$. CO_2 levels in both influent and effluent gas flows were routinely measured at steady state and corrected for ambient temperature and water vapor pressure. Particulate carbon and nitrogen were measured on a Perkin Elmer 240 elemental analyzer. Cells were counted in a Spencer Bright-line hemacytometer. Dry weights were determined on 100 ml samples retained on pre-combusted glass-fiber filters and combusted at $500-500^\circ\text{C}$ for >4 hours. Culture and medium pH was measured with a combination probe mounted on a Corning 110 meter. All measurements were made directly on culture samples at the steady state, defined as the time when culture absorbance, measured on a Bausch and Lomb Spectronic 88 at 600 nm, did not vary more than $\pm 10\%$ for at least 2 consecutive days.

3. Results

a. HCO_3^- Experiments: There was a common linear response in the mass flux of algal carbon produced with increasing mass inputs of HCO_3^- alkalinity, but only within restricted pH ranges. For P. tricornutum up to 25 mg C/day of algal carbon was produced when culture pH values were <9 , representing an efficiency of carbon assimilation of 100 (Fig. 2A). For the 3 freshwater chlorophytes the efficiency of assimilation was 72 with up to 25 mg C/day of algal carbon produced at $\text{pH} < 8$ (Fig. 2B). Maximum concentrations of added inorganic carbon that led to linear responses in algal production were respectively 109 mg C/liter in the seawater medium and 135 mg C/liter in the freshwater medium. Above these concentrations at any pH and at pH values greater than and HCO_3^- additions lower than noted above, algal productivity was reduced significantly below the linear responses. In some experiments, particularly at the higher HCO_3^- concentrations, chemical precipitates were observed, leading to progressive deterioration of the cultures.

The amount of algal carbon per unit of biomass, expressed as the C:dry wt ratio (mg:mg), was remarkably constant for all 4 species at $\sim 0.45\text{--}0.50$ when growth occurred in the respective pH ranges that represented linear relationships between productivity and mass input fluxes of HCO_3^- alkalinity (Table 1). There was, however, a slight increase in the C:N ratio from 5.04 for P. tricornutum to 6.75 for the S. obliquus, with the other two chlorophytes displaying intermediate C:N ratios (Table 1).

b. Bubbled Gas Experiments

i. Bubble size:

At each CO_2 level in the range 0.036% (air) to 0.167% there was a virtual linear increase in algal productivity with increase in the specific gas bubbling rate G over a wide range of input fluxes for the large bubbles (Fig. 3). In contrast, algal productivity, although greater with small bubbles for a given CO_2 level at the lower bubbling rates, led to growth inhibition at the higher values of G (Fig. 3). The value of G leading to this inhibition appeared to decrease with increasing CO_2 - from ~125/hr at 0.036% CO_2 to ~50/hr at 0.167% CO_2 .

The dramatic effect of bubble size on productivity can be seen in Figure 4 in which the data have been plotted on a mass flux basis. For both large and small bubbles there was a linear relationship between flux in and flux out. For large bubbles the efficiency of assimilation was only 14%, whereas for the small bubbles the efficiency increased to 47%. However, the impact of small bubble inhibition at the higher values of G was readily apparent at an input mass flux >300 mg C/day.

Through a mass balance comparing the fluxes of bubbled inorganic carbon into and out of the culture with algal productivity for 0.036% CO_2 (Fig. 5), it was estimated that <7% of the input inorganic carbon was unaccounted for, possibly representing excreted dissolved organic carbon which was not measured.

ii. $K_L a$ and bubble rate

From eq. (14) it follows that for a given set of environmental conditions and constant culture volume, the mass transfer coefficient $K_L a$ should be linearly proportional to the bubble rate and independent of bubble size (dashed line in Fig. 6). $K_L a$, determined from experimental data using eq. (11) for 0.036% CO_2 , compared extremely well with the theoretical relationship (Fig. 6).

iii. Enriched CO_2

When the cultures were maintained on 1% CO_2 at very low bubble rates, the relationship between carbon flux in and out was independent of bubble size and was virtually identical to the flux curve established with small bubbles and $\text{CO}_2 \leq 0.167\%$ (Fig. 7). However, at input fluxes somewhat greater than 300 mg C/day the output flux of algal carbon reached a saturation level of ~225 mg C/day (Fig. 7).

Assimilation efficiencies up to 61% (small bubbles) and 38% (large bubbles) were attained with the combination of 1% CO_2 and input fluxes ≤ 350 mg C/day. With further increases in input flux to ~3000 mg C/day (equivalent $G=50/\text{hr}$), independent of bubble size, there was no increase in productivity above ~225 mg C/day (~8 efficiency) (Fig. 7); moreover when G was increased to $\geq 120/\text{hr}$ productivity decreased to <200 mg C/day (Fig. 7). Thus at the highest input fluxes used (~10,000 mg C/day) assimilation efficiencies were reduced to 2 .

When the CO₂ level was raised to 100% after the cultures had reached steady state on 1% CO₂, cell washout occurred regardless of the bubble rate in the range 2-125/hr.

iv. Nitrogen and light limitation

In several experiments with S. obliquus it was observed that virtually 100 of the medium NH₄⁺ (56-112 mg N/liter) was assimilated depending on the input mass flux of inorganic carbon. (Table 2). When this occurred algal productivity was diminished to ~150 mg C/day and a saturation level which defined nitrogen limitation was established that was considerably less than the light saturation plateau that resulted when an excess of nitrogen was supplied (Fig. 7). In addition, for a given level of limiting influent nitrogen, as the input carbon flux and/or efficiency of use increased and complete nitrogen assimilation occurred there was a dramatic increase in the algal C:N ratio from <6 to >10. In all cases, however, the cellular carbon constituents remained constant at ~20 pg C/cell and 0.5 mg C:mg dry weight, irrespective of the degree of nitrogen limitation, indicating that changes in the C:N ratio were due to changes in cellular nitrogen constituents (Table 2).

In two cultures grown on 1% CO₂ and in which ample nitrogen was present algal productivity was considerably less than predicted by the light limitation plateau in Figure 7. These two cultures were positioned at the end of the bank of eight cultures near to the end of the fluorescent bulbs, where the light intensity was found to be about 60% of the intensity at the center of the culture bank. Once the entire culture

units were switched to the center of the bank, productivity increased dramatically to the region of higher light limitation experienced by the other cultures (arrows in Fig. 7).

v. CO₂-helium mixtures

In one experiment duplicate cultures were grown on 1% CO₂-99 helium to test if oxygen toxicity might be a factor in reducing algal productivity. As seen in Figure 7, no enhancement in productivity was observed over 1% CO₂-99 air.

vi. Dilution rate

For conditions of large bubbles, 0.036% CO₂, and G=100/hr, there was an exponential decrease in the steady state concentration of algal carbon with increasing dilution rate until complete biomass washout occurred at D = 1.25/day (Fig. 8). As a result, peak algal productivity of ~38 mg C/day occurred at a dilution rate of ~0.5/day.

viii. Low pH

In one experiment with C. vulgaris grown on 0.036% CO₂ and large bubbles at low bubbling rates (G=8/hr) the buffer was eliminated from the medium and culture pH values dropped to ~4.5 as NH₄⁺ uptake led to concomitant production of hydrogen ions. Under these conditions algal productivity was identical to that in buffered cultures.

4. Discussion

A major conclusion from this study is that HCO₃ alkalinity, although

an excellent source of inorganic carbon in terms of assimilation efficiency, can provide only limited quantities of inorganic carbon to algal mass cultures compared to bubbled CO_2 . As seen in Figure 9, even though the efficiency of carbon assimilation from HCO_3^- alkalinity was extremely high (72-100 %), and steady state algal biomass was predicted by eq. (7) very well, problems related to pH control and associated chemical precipitation of a potentially wide variety of salts of CO_3^{2-} , OH^- and PO_4^{3-} at high pH and alkalinity¹⁹ probably were the major factors limiting productivity to < 28 mg C/day.

As shown previously^{8,20}, and as verified here, the rate reactions in the $\text{CO}_2\text{-HCO}_3^-\text{-CO}_3^{2-}$ are not limiting steps in the overall supply of inorganic carbon from the aqueous phase to the sites of photosynthesis within an individual cell. Otherwise, linearity between C_A and C_{T_0} could not be established. Moreover, the actual affinity for inorganic carbon at the cell surface has been shown by Markl²¹ to be astonishingly low, ca. $\leq 1 \mu\text{g C/liter}$ for *C. vulgaris*. Hence, for the case in which HCO_3^- alkalinity in the major source of inorganic carbon for algal growth, increases in pH and/or alkalinity lead to chemical deterioration of the growth medium and place an upper limit on productivity. This upper limit is considerably below the potential limit that would result from light limitation. For example, in the current work there was almost a 10-fold increase in productivity when bubbled CO_2 replaced HCO_3^- as the carbon source and the light intensity was held constant (Fig. 9).

Clearly, bubbled CO_2 must be provided to force light to be the sole limiting growth factor. From both an economic and technical standpoint, however, the problem is far more complex than simply providing an excess of bubbled CO_2 . As seen in Figures 3 and 4, and as would be expected, small bubbles, because of their larger surface area per unit volume, are significantly more efficient than large bubbles in supplying CO_2 to the surface of an algal cell where consumption occurs, at least at CO_2 levels $\leq 0.167\%$. Yet, an interesting outcome of the mass balance eqs. (8) and (12) is that the overall mass transfer coefficient $K_L a$, as defined in eq. (14), simply is a linear function of specific bubbling rate G , and is not related to bubble size (Fig. 6). Within the range of G employed $K_L a$ varied from $\sim 0.1/\text{min}$ to $\sim 3/\text{min}$, values considerably less than found for *C. vulgaris* ($\sim 11/\text{min}$) by Markl²¹. However, $K_L a$, as used in this study, is not a measure of the efficiency of physical mass transport of CO_2 from the gas phase to the cell surface, as considered by Markl;²¹ rather, because of the imposed steady state carbon limitation, it is an indicator of the mass flux of inorganic carbon available to the culture. The actual efficiency of carbon use for a given influence CO_2 level is a function of P_1 , the steady state residual CO_2 concentration in the culture, because all other parameters in eq. (11) are not dependent on bubble size. Hence, for the current investigation, the slopes of the mass flux input-output curves in Figure 4 provide the only useful information on assimilation efficiencies in relationship to the total amount of inorganic carbon made available to the cultures.

When inorganic carbon is limiting growth, the only determinant of productivity is the mass flux of carbon introduced to the cultures, which is the product of bubbles rate and CO_2 . Thus, as seen in Figure 4, and predicted by eq. (11), various combinations of G and CO_2 within certain ranges lead to the same productivity for a given dilution rate. And for a fixed CO_2 concentration in the influent gas the relationship between productivity and G is linear (Fig. 3). Both Hannon and Patouillet²² and Ammann and Lynch²³ found a similar dependency of mass input flux on the rates of photosynthetic O_2 evolution in Chlorella pyrenoidosa.

Theoretically, productivity should increase linearly with increasing mass input flux of carbon until light limitation occurs. By using high levels of CO_2 (~1%), it was possible to reach light limitation at very low bubbling rates (Fig. 7) and still maintain high assimilation efficiencies. Moreover, with 1% CO_2 the degree of efficiency was independent of bubble size, probably because the level of P_1 in the culture was always high enough to prevent any mass transport bottlenecks between the gas phase and the cell surface. From the standpoint of optimizing productivity, either a combination of low CO_2 level and high bubble rate (with small bubbles) or one with high CO_2 level and low bubble rate (with large or small bubbles) should give comparable results. However, the inhibition observed at the higher bubble rates when small bubbles were used (Fig. 3) places a restriction on obtaining high productivity with small bubble aeration (Fig. 9). It is difficult to hypothesize as to the cause of this inhibition; however, some frothing at the culture surface was noted in the small bubble experiments, possibly leading to the phenomenon of

froth flotation in which algal cells adhere to small bubbles and accumulate at the surface where they are selectively washed out.²⁴ In retrospect, by simply replacing the surface overflow port on the culture unit with a submerged overflow, small bubble inhibition might have been avoided. From an economic standpoint, however, small bubble aeration might not be attractive due to problems of clogging and associated friction losses. Use of enriched CO₂, low bubble rates, and large bubbles appears to be the most attractive method for optimizing the supply of CO₂ to mass cultures.

As seen in the summary curves of Figure 9, the optimum input flux for the current study was ~450 mg C/day, assuming that the slope of the efficiency curves for 1% CO₂ and $\leq 0.167\%$ CO₂ (small bubbles) were similar. Any additional CO₂ added to the cultures was wasted. At higher light intensities additional CO₂, either by increasing CO₂ or G, would be necessary to raise productivity to the corresponding light limiting level (Fig. 9).

The decrease in productivity at 1% CO₂ but with $G \geq 120/\text{hr}$, independent of bubble size (Fig. 7), does not seem to be a result of the same type of inhibition observed at the lower CO₂ levels with small bubbles. In the latter case the bubbling rate causing this inhibition seemed to decrease from ~120/hr at 0.036% CO₂ to ~50/hr at 0.167% CO₂ (Fig. 3), suggesting some effect of the CO₂ tension on the degree of cell flotation. Yet, when 1% CO₂ was used the inhibition was manifested only at $G \geq 120/\text{hr}$. At such high bubbles rates and concomitant total input fluxes, the influent and effluent CO₂ levels were identical at 1% so that a true

narcotic effect on algal growth at high CO_2 may have led to the above inhibition. Moreover, when 100% CO_2 was used over a wide range of bubble rates complete death occurred. For even at the lowest bubble rate ($G = 2/\text{hr}$) $>10,000 \text{ mg C/day}$ was supplied, most likely resulting in an exceedingly high CO_2 level at the cell surface. Others have observed similar narcotic effects of CO_2 levels ≥ 1 .^{23,25,26} As pointed out by Steemann Nielsen,²⁶ and confirmed by Ammann and Lynch,²³ the critical factor in analyzing the narcotic effect of high ($>1\%$) CO_2 on algal growth is the actual CO_2 level in contact with individual cells. The magnitude of the CO_2 tension at the cell surface is determined by the combination of influent CO_2 level and bubbling rate (mass in) and the demand for carbon determined by the combination of concentration of algae and growth rate (mass out). Hence, it is important to maintain a tight balance between input and output fluxes, not only to prevent wastage of CO_2 , but also to maintain a low residual CO_2 concentration in solution and avoid CO_2 narcosis.

The control of CO_2 tension at the cell surface (the difference between mass flux in and out) may explain why in two previous studies 100% CO_2 was successfully used to culture Chlorella sp.^{27,28} In both studies 100% CO_2 was supplied on demand, in one case by a pH-stat system to control pH,²⁷ and in the other by a recirculating growth system in which the mass production of algal carbon purposely was balanced to the mass input of CO_2 .²⁸ In the former case the actual CO_2 tension in solution, although not measured, probably was lower than toxic levels, and in the latter case there appeared to be a direct relationship between

increases in culture CO_2 beyond a few percent and decreases in productivity. To date, little quantitative data are available on species-specific narcotic effects of high CO_2 in microalgae, although there seems to be little doubt that the effect is real.

In contrast, there was no apparent toxic effect of oxygen on productivity, as demonstrated by the experiments in which helium was used in place of air in preparing the 1% CO_2 mixtures (Fig. 7). High oxygen levels can lead to photorespiration²⁹ and/or photooxidation,³⁰ but apparently only when % CO_2 is low and % O_2 is >20%.³¹ Continuous bubbling with enriched CO_2 , as practiced in the current study, more than likely prevents any increase in % O_2 above saturation levels so that oxygen toxicity should not be a common problem in well-mixed cultures.

In large-scale practice, the most expedient method for simultaneously providing adequate CO_2 , minimizing the solution CO_2 level, and controlling pH probably is through a pH-stat system in which bubbled CO_2 -enriched air is added on demand as algal growth causes a pH rise above a desired level via reactions (1) and (2). In the current study pH arbitrarily was controlled in the range 6-8 for the freshwater algae and 8-9 for P. tri-cornutum with the aid of aqueous buffers, which would be prohibitively expensive in real practice. Identification of species-specific tolerances to changing pH was beyond the scope of this study. However, chemical precipitation problems were observed at pH > 8 in the alkalinity studies which obscured any possible physiological effect of high pH on growth. However, the freshwater algae at least seemed to tolerate low pH down to ~4.5 without any decrease in productivity. These results are consistent

with those of Emerson and Green³² who found no effect of pH in the range 4.6-8.9 on photosynthetic rates in both C. vulgaris and C. pyrenoidosa. Further research will be necessary to determine the optimum pH for maximizing productivity and minimizing CO₂ losses.

Under conditions of nitrogen limitation, not only is productivity curtailed (Fig. 7), but dramatic increases in the cellular C:N ratio occur (Table 2). These results vividly demonstrate the need to supply excess nutrients in addition to CO₂ to force light to become limiting. However, in certain situations it may be desirable to alter the cellular chemical composition to produce cells high in lipids and hydrocarbons.³³ Forcing a nitrogen limitation on the culture may be a technique for maximizing production of such compounds with only a small sacrifice in total yield.

Under all conditions of light or carbon limitation with either HCO₃⁻ or CO₂ as carbon sources the cellular chemical composition of all 4 species were invariant: cellular C:N ratios were 5-7 (mg:mg) and the C:dry wt ratios were ~0.45-0.5 (Tables 1 and 2). Such ratios represent well-nourished cells containing ~50% protein.³⁴ Similar results were obtained in previous carbon limitation studies under a wide range of growth rates.^{8,20,35}

The decrease in steady state algal concentration with increasing dilution rate under carbon-limiting conditions (Fig. 8) is identical to that found by Pipes.³⁵ This response is predicted by eq. (11) for conditions of constant G and P_O; and, as demonstrated in Fig. 8, it is an important consideration in determining the optimum dilution rate for maximizing productivity.

5. Conclusions

Optimization of inorganic carbon supply to mass algal cultures involves not only basic mass transport considerations such as culture geometry, bubble size, gas flow rate, and P_{CO_2} , but also an understanding of the physiological responses to the various combinations of these parameters. From an economic standpoint, the main objective is to maximize productivity and carbon assimilation efficiencies simultaneously with the combination of optimum growth conditions and most economical CO_2 supply system. The results of this study, hopefully demonstrate a rationale framework for meeting this objective. The efficiencies, yields, and constraints reported here are only valid for the culture system and growth conditions employed in this study and should not be extrapolated for designing large-scale mass culture systems. The concerns over bubble size, high bubble rate and CO_2 toxicity undoubtedly will be common to all growth situations, though. Moreover, total input flux, which is the product of gas bubbling rate and CO_2 , will be the major yield determinant in both laboratory and outdoor cultures. Selecting the proper combination of these two variables is the key to avoiding carbon limitation in intense cultures.

6. References for Section C

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7. Tables and Figures for Section C.

Table 1. Cellular chemical ratios for freshwater and marine algae grown in inorganic carbon-limited continuous cultures with HCO_3^- alkalinity as sole carbon source at a dilution rate of 0.5/day.

Species	No. Datum Points	pH Range	C:N Ratio (mg:mg)	C:Dry Wt Ratio (mg:mg)
<u>Phaeodactylum tricornutum</u>	28	7.8-9.0	$5.04 \pm 0.42^*$	0.49 ± 0.07
<u>Chlorella vulgaris</u>	11	6.9-7.7	5.51 ± 0.41	0.44 ± 0.05
<u>Selenastrum capricornutum</u>	4	6.9-7.7	5.87 ± 0.29	0.50 ± 0.02
<u>Scenedesmus obliquus</u>	15	6.9-7.9	6.75 ± 0.87	0.45 ± 0.06

* Standard deviation

Table 2. Effect of influent ammonium concentration and gas bubble %CO₂ on cellular C:N ratio and cellular carbon for Scenedesmus obliquus grown in continuous cultures at a dilution rate of 0.5/day and a specific gas bubble rate of ~140/hr.

Bubble Size	CO ₂ o/o	Medium NH ₄ ⁺ Conc. (mg N/liter)	Algal Nitrogen Conc. (mg N/liter)	Algal Carbon Flux out (mg C/day)	Algal C:N Ratio (mg:mg)	Cellular Carbon	
						(pg C/cell)	(mg C:mg dry wt)
Large	0.036	56	44	53	5.6	-	0.51
Small	0.036	56	56	108	7.4	17.7	0.52
Large	0.9	56	58	151	10.5	20.5	0.51
Small	1.0	56	57	144	10.3	21.5	0.49
Large	0.036	112	44	54	5.5	14.5	0.47
Small	0.036	112	75	105	5.8	19.3	0.53
Large	1.0	112	120	197	7.1	19.8	0.52
Small	1.0	112	87	138	6.1	18.7	0.51
Small	1.0	112	113	207	7.3	18.1	0.54
Small	1.0	176	132	188	5.7	32.2	0.52

Figure Legends

- Figure 1. Schematic diagrams of continuous culture system for inorganic carbon studies: 1- medium feed bottle, 2- ice-filled styrofoam container, 3- peristaltic feed pump, 4- feed lines, 5- siphon break, 6- 0.5 liter glass culture vessel, 7- row of six - 40W fluorescent lamps, 8- overflow line, 9- overflow collecting bottle, 10- teflon-coated magnetic stirring bar, 11- magnetic mixer, 12- laboratory air line, 13- CO₂ cylinder, 14- gas proportioner, 15- gas flowmeter, 16- gas line to culture, 17- gas exhaust port, 18- circulating temperature control bath.
- Figure 2. Relationship between inorganic carbon flux in as HCO₃⁻ alkalinity and algal carbon flux out of continuous culture at fixed dilution rate of 0.5/day. Dashed lines represent 100 efficiency. A, P. tricornutum: 0-pH < 9, 0-pH > 9; B, Freshwater algae: closed symbols - pH < 8, open symbols - pH > 8, 0, 0 - S. obliquus, Δ, Δ - C. vulgaris, , - S. capricornutum. Solid line represents linear regression curve, correlation coefficient $r = 0.95$.
- Figure 3. Relationship between specific gas bubble rate and algal carbon mass flux out of continuous culture for S. obliquus at fixed dilution rate of 0.5/day and varying CO₂ levels in bubbled gas.
- Figure 4. Relationship between bubbled CO₂ mass flux in and algal carbon mass flux out of continuous culture at fixed dilution rate of 0.5/day for %CO₂ levels in bubbled gas <0.167. Open symbols-small bubbles ($r=0.95$) closed symbols-large bubbles ($r=0.97$); o, o - S. obliquus, Δ - C. vulgaris, - S. capricornutum.
- Figure 5. Relationship between bubbled CO₂ mass flux balance (in-out) and algal carbon mass flux out of continuous culture for freshwater algae at fixed dilution rate of 0.5/day and 0.036% CO₂ in bubbled gas ($r=0.97$): o - S. obliquus, Δ - C. vulgaris, - S. capricornutum. Dashed line-theoretical 100% efficiency.
- Figure 6. Relationship between specific gas bubbling rate and mass transfer coefficient at dilution rate of 0.5/day and 0.036 %CO₂ in bubbled gas ($r=0.97$): Open symbols - small bubbles, closed symbols - large bubbles; o, o - S. obliquus, Δ - C. vulgaris, - S. capricornutum. Dashed line represents theoretical relationship from eq. (14).

- Figure 7. Relationship between bubbled CO_2 mass flux in and algal carbon mass flux out of continuous culture at fixed dilution rate of 0.5/day for 1% CO_2 in bubbled gas for growth of S. obliquus. Open symbols - small bubbles, closed symbols - large bubbles: o, o - light limited, Δ, Δ - nitrogen limited, o - low light limited S. obliquus cultures (arrows refer to increased productivity when light level was increased by repositioning culture from end to center of fluorescent bulbs), - S. obliquus grown in 1% CO_2 -99% helium.
- Figure 8. Relationship between specific growth rate (dilution rate, and both steady state algal carbon (broken line) and algal mass flux out (dashed line) for growth of S. obliquus in continuous culture with large bubbles, specific bubble rate = 100/hr and 0.036% CO_2 .
- Figure 9. Summary curves for algal productivity as a function of source of inorganic carbon: 1- theoretical curve representing 100% efficiency; 2- curves for HCO_3^- alkalinity as sole source of inorganic carbon (no gas phase) and $\text{pH} < 8$; 3- bubbled gas (large bubbles) as sole inorganic carbon source and $< 0.167\%$ CO_2 ; 4- bubbled gas (small bubbles) as sole inorganic carbon source and $< 0.167\%$ CO_2 (curve ends at $\sim 300 \text{ mg C day}^{-1}$ input); 5- bubbled gas² (irrespective of bubble size) as sole inorganic carbon source for 1% CO_2 ; 6- light limitation plateau for light intensity used in experiments; 7- increased productivity as function of increased light intensity.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

D. THE EFFECT OF pH ON BIOMASS REGULATION

1. 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 nonaxenic mass cultures of desired species by weed microalgae such as the chlorophyte Scenedesmus sp. in freshwater systems and the marine diatom Phaeodactylum tricornutum in marine counterparts (Oswald and Golueke, 1968; Goldman and Ryther, 1976). Goldman (1976) speculated that among a group of marine species possessing equal growth characteristics in intensive culture, P. tricornutum often is the successful competitor because of its ability to tolerate high pH or excrete toxic compounds (allelopathy), or both; however, as shown by Goldman and Ryther (1976), this competitive edge occurs only within a restricted temperature range of $\sim 10^{\circ}$ - 20° C.

To date, the role of allelopathy in competition between P. tricornutum and other marine algae is poorly defined. On the one hand, Sharp et. al. (1979) claimed that the ability of P. tricornutum 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 P. tricornutum to outgrow T. 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 P. tricornutum dominated in mixed cultures the pH was uncontrolled; moreover, in those studies which contained data on pH (Goldman and 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. tricornutum became the major species. In addition, one of us (Goldman, 1976) observed that the highest pH attained in continuous monocultures of P. tricornutum consistently was >10, whereas in similar cultures of T. pseudonana (3H) the pH never rose above ~9 and in cultures of Dunaliella tertiolecta it did not exceed ~9.4.

These results were consistent with the findings of Humphrey (1975), who showed that P. tricornutum was among a small group of marine algae that could tolerate pH values over 10. Hence, we concluded that there was a strong circumstantial argument in support of a role for pH control over species dominance and that further research on this topic was necessary.

Often it is difficult to interpret results from pH studies because of problems in separating the effects of high pH on cell physiology from those due to pH-mediated changes in aqueous chemistry. For example, both the chemical speciation of inorganic carbon sources and the availability of sparingly-soluble nutrients are affected greatly by pH (Goldman, 1973). In this study we examine the role of pH on biomass regulation in intensive cultures of both marine and freshwater microalgae, independent of inorganic carbon limitation. In Section F we investigate impact of pH on species competition.

2. Materials and Methods

a. Test algae: We obtained cultures of Phaeodactylum tricornutum (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 T. pseudonana (3H) for the competition studies with P. tricornutum mainly to avoid any possibility that silicon limitation could bias the results. Both P. tricornutum and D. tertiolecta do not require silicon, whereas T. 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.

b. Nutrient media: Medium for the marine studies was synthetic seawater containing 400 mM NaCl, 20 mM MgCl₂, 20 mM MgSO₄, 10 mM CaCl₂, 10 mM KCl, 0.8 mM KBr, 0.2 mM H₃BO₃, 2 mM NaHCO₃, 5 mM NaNO₃, 0.5 mM KH₂PO₄, and vitamins and trace metals plus the sodium iron salt of ethylenediamine-tetraacetic acid (EDTA) in a two-fold dilution of the amount in f-medium (Guillard and Ryther, 1962).

The freshwater medium contained 0.4 mM MgCl₂, 0.4 mM MgSO₄, 0.2 mM CaCl₂, 2 mM NaHCO₃, 12 mM NaNO₃, 1 mM KH₂PO₄ and the same concentrations of EDTA-trace metals as in the seawater medium.

c. Culture system and pH control: The continuous-culture apparatus (a bank of seven 0.5-liter cultures), the culturing protocols, and the experimental analyses virtually were identical to those described previously (Goldman et al., 1981). Continuous lighting ($0.06\text{--}0.07\text{ cal}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$), 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 $0.45\text{--}0.60\text{ day}^{-1}$ 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 $7.6\text{--}10.6$ with a pH-stat system, identical in design to those of Soltero and Lee (1967) and Sperling et al. (1974), that provided sufficient CO_2 -enriched air ($1\%\text{CO}_2$) 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_2 -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 units from the set value. At the start of an experiment when algal biomass was increasing to a steady state level, 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_2 , as regulated by the pH controller. In this manner, we insured 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 CO_2 .

We obtained a mixture of 1% CO_2 in air by blending 100% CO_2 from a pressurized cylinder with laboratory air in a 2-gas proportioner. The flow rate of gas to each culture was held constant at $0.7 \text{ liters} \cdot \text{min}^{-1}$ with a rotometer on the gas inlet. The total amount of 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% CO_2) into the culture at a rate of $0.7 \text{ liters} \cdot \text{min}^{-1}$. 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).

d. Culture operation: e 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 - 10.6. When steady state was reached the cultures were sampled for cell counts, particulate carbon and nitrogen.

e. Analyses: Particulate carbon and nitrogen were measured on a Perkin Elmer 240 elemental analyzer. Cell counts were made on a Spencer Bright-line hemocytometer. Steady state growth rates μ were equal to D (Goldman, 1977). Thus the maximum tolerable pH for each species was defined as the pH above which μ could not be sustained at 0.5 day^{-1} and cell washout ensued.

3. Results and Discussion

a. Lower pH limits: We found that it was impossible to establish pH values lower than 7.6 in the marine growth medium and 7.9 in the fresh-water medium (Table I). These lower pH limits are not the pH levels that the test algae were capable of tolerating, but rather represented the respective equilibrium pH values that resulted when the partial pressures of CO_2 in the gas and liquid phases came into equilibrium. The equilibrium pH is controlled, not only by the partial pressure of CO_2 in the gas phase, which in this case was 0.01 atm, but also by the alkalinity (Stumm and Morgan, 1970). In the present experiments the alkalinity was variable and increased above the initially added 2 meq HCO_3^- in direct proportion to the amount of NO_3^- assimilated by the algal cultures (Osterlind, 1949; Brewer and 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% CO₂-enriched gas was supplied continuously at a flow rate of 0.7 liters·min⁻¹. This flux of inorganic carbon into the culture vessel was in great excess relative to the growth requirements of the algal cultures (Goldman et al., 1981), so that CO₂ equilibrium between the gas and liquid phases was possible. The pH under the above conditions was determined indirectly by the steady state concentration of algae because alkalinity increase was proportional to biomass production.

Many freshwater eukaryotic algae are unaffected by acidic pH (Brock, 1973). Both S. obliquus and C. vulgaris belong to this group of acidophilic algae and can tolerate pH values down to 4 or less (Emerson and Green, 1938; Osterlind, 1949; Goldman et al., 1981). Similarly, although marine algae do not display acidophilic characteristics, both P. tricornutum and D. tertiolecta have been cultured successfully at pH values as low as 6 (Hayward, 1968; Humphrey, 1975). Therefore, our inability to attain steady state pH levels below 7.6 for the marine algae and 7.9 for the freshwater algae was not due to a physiological limitation, but rather was a clear demonstration of how the source of nitrogen, though biologically-mediated alterations in the aqueous buffer system (in this case OH⁻ production balancing NO₃⁻ uptake) was the main determinant in establishing the lowest culture pH possible. As will be shown elsewhere (Goldman et al., in prep.), the selection of a culture pH that will allow maximum productivity and at the same time provide for the most economical supply of inorganic carbon, is dependent on the regulation of both nitrogen source (for controlling alkalinity) and the percentage CO₂ in the bubbled gas.

b. 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 NO_3^- and PO_4^{3-} concentrations in the marine growth medium to 5 mM and 0.5 mM, respectively to insure culture stability, but at the expense of allowing the cultures to become depleted in 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 (Fig. 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.

In addition, by fixing the dilution rate at 0.5 day^{-1} , the maximum tolerable pH exhibited by each culture simply reflected the highest pH for which a steady state μ of 0.5 day^{-1} could be maintained, and is not necessarily a measure of the absolute pH maxima possible for the test species. Under these conditions the highest pH levels attained were ~ 10.6 for the freshwater species, ~ 10.3 for P. tricornutum, and ~ 9.4 for D. tertiolecta (Table I). In all cases the pH maxima were the same regardless of whether the pH was regulated by the pH-stat system or it was allowed to increase via continuous bubbling of air without external control (Figs. 2 and 3).

These results, nonetheless, are consistent with the general findings that many freshwater chlorophytes and diatoms exhibit maximum photosynthesis over a broad pH range and can tolerate pH values as extreme as 10.5 to 11 and greater (Emerson and Green, 1938; Osterlind, 1949; Felfoldy, 1962; Moss, 1973; Talling, 1976). In contrast, although data on pH responses by marine algae are limited, a large number of 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 seawater which is ~8.1 to 8.3 (Kain, 1958 a,b; 1960; Hayward, 1968; Humphrey, 1975). Yet, a few marine species, particularly P. tricornutum, seem to behave more like 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, 1958 a,b; 1960; Hayward, 1968).

There are no clearcut reasons why freshwater chlorophytes should be capable of tolerating a much wider range in pH than marine species. Intuitively, it seems reasonable to invoke the hypothesis 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 less than 4, and do not, in general, grow well even in mildly acidic environments (Brock, 1973). In contrast, natural marine algae, having

little opportunities for experiencing pH levels that deviate even slightly from the well-buffered pH of seawater, probably never have evolved any physiological potential for thriving in extreme pH environments. The unique ability of P. tricornutum to grow well at pH 10 thus is amazing considering that it is never found in abundance in natural seawater populations under any environmental conditions (Harvey, 1955).

In most studies dealing with pH effects on algal growth, it has been extremely difficult to separate true physiological responses to extreme pH from ones related to alterations in the chemical species of inorganic carbon and other sparingly-soluble nutrients (e.g. phosphorus and trace metals) (Goldman, 1973). We are confident, however, that the upper pH limits we observed were not due to a decrease in the availability of free CO₂ at high pH. First, for each species the supply of inorganic carbon was a dependent variable, controlled solely by the photosynthetic demand for carbon at a particular pH. In this manner, as long as a species could grow at a given pH, the flux of CO₂ into the culture was proportional to the demand. Second, the pH maxima attained in the continuous air-grown cultures generally were no higher than the respective pH maxima achieved under pH-stat control (Figs. 2 and 3). This occurred even though the total flux of inorganic carbon was substantially different with the two systems - continuous input of air (0.036% CO₂) versus 1% CO₂ supplied on demand. In the case of S. obliquus the different modes of CO₂ supply led to gross differences in steady state biomass (Fig. 3A) and concomitant differences in culture alkalinity, dissolved inorganic carbon and free CO₂ at the common pH maxima of ~10.6 (Table II). In consideration of these results, any form of inorganic carbon limitation seems unlikely.

Other explanations for the observed pH maxima, such as inhibition of physiological processes or alterations in the availability of sparingly soluble nutrients at high pH can not be excluded, but are very difficult to confirm. Nonetheless, with the exception of the results with D. tertiolecta, there is some evidence to support the possibility that the response to high pH primarily was a metabolic one. First, the two species that were least affected by changing pH, S. obliquus (Fig. 3A) and P. tricornutum (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 also was dramatically evident in the air-grown cultures of S. obliquus. 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). Because pH control in this case was directly linked to cell physiology, this oscillation may have been caused by a metabolic feedback mechanism that was manifested as a decrease in μ below 0.5 day^{-1} (the steady state dilution rate) when the threshold pH was exceeded. This led to cell washout and a concomitant decrease in pH. When the pH dropped below an inhibitory level of about ~ 10.6 , the restraint on μ was lifted leading to an increase in cell biomass and a rise in pH to complete the cycle. The rather large amplitude in this cycle (~ 1 - 1.5 pH units) may

reflect a relatively sluggish feedback mechanism in this alga. Conversely, our inability to observe any oscillations in pH in air-grown cultures of the other species, particularly for those algae capable of tolerating high pH (i.e. C. vulgaris and P. tricornutum), simply may mean that the feedback inhibition mechanism for many algae is so finely tuned that oscillations around the threshold pH are too small to detect.

Still, the possibility can not be discounted that chemical alterations to the growth media at high pH was the major determinant of each species' tolerance to pH. This is particularly true of D. tertiolecta and many other marine algae, which do not grow at $\text{pH} > 9.3$. Due to the presence of large amounts of bivalent cations such as Ca^{+2} and Mg^{+2} in seawater, 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 P. tricornutum to tolerate pH values above 9.3 simply may reflect a reduced requirement for an essential nutrient that becomes less available with increasing pH (Stumm and Morgan, 1970). 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 NO_3^- and 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.

c. 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). However, when the pH was increased into the alkaline region, we observed two distinctly different types of response. On the one hand, both P. tricornutum (Fig. 2A) and S. obliquus (Fig. 3A) were relatively unaffected by varying pH, and biomass levels for both species varied by no more than ~30% over the range of pH tested. On the other hand, D. tertiolecta (Fig. 2B) and C. 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 P. tricornutum should be successful in intensive marine cultures that are poorly buffered. Clearly, once the pH rises above ~9.3 P. tricornutum can grow without competition from species such as D. tertiolecta because of the latter's inability to tolerate high pH. Pruder and Bolton (1979) found the same sensitivity to high pH in T. pseudonana (3H). Thus the repeated dominance of P. tricornutum in large-scale outdoor cultures may be linked to poor pH control. However, as we will demonstrate in Part II of this study (Goldman, et al. in prep.), pH control alone is not the solution to maintaining other more desired species in culture.

Even though there were significantly different growth responses among the test species 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 1). Moreover, the average values for these cell quotas are indicative of non-nutrient limiting conditions, when compared with results on the individual species from previous studies that dealt with nutrient kinetics (Goldman, 1976; Goldman and Peavey, 1979; Goldman and Mann, 1980; Li, 1980; Goldman and Graham, 1981). Similarly, the constant and low cellular carbon:nitrogen ratios of 5.0 to 6.5 (by weight) represent algal cells in a nutritionally well-balanced state (Goldman, 1980).

Therefore, it would appear that variations in pH have little effect on the distribution of macromolecular components in algal cells, even though such changes undoubtedly result in gross alterations of certain metabolic functions. For example, Smith and Raven (1979) suggest that under conditions of varying external pH, intracellular regulation of pH is a basic physiological function of plant cells (including microalgae) that involves complex processes of membrane transport and intracellular metabolism. Also, Malis-Arad et al. (1980) demonstrated that cell aggregation of C. vulgaris is caused by high pH-induced increases in both cell pectin content and cell volume. This then leads to incomplete cell division and the formation of clusters of autospores attached to the cell walls of mother cells. However, even at high pH, pectin material in C. vulgaris represents <2% of total cell dry weight (Malis-Arad et al., 1980), so that its contribution to changes in total cellular carbon virtually would be undetectable.

4. Conclusions

The role of pH in regulating biomass and species composition appears to be more important in marine mass cultures than in freshwater counterparts. The major reason for this difference is that most marine algae do not grow well (or at all) at pH values greater than 9 to 9.5; thus the few species that are capable of tolerating higher pH, such as P. tricornutum, grow unrestricted in poorly buffered medium. Under these circumstances the pH is regulated internally by biological activity and, hence, rises as high as is dictated by the alga's upper tolerance to pH. Aqueous chemical processes controlling the solubility of essential, but sparingly soluble nutrients most likely are major factors in setting pH limits of 9 to 9.5 for the bulk of marine species. For freshwater situations the role of pH is not so clearcut because the ability to tolerate extreme pH (>10.5) is a common characteristic of many freshwater species and precipitation of essential nutrients at high pH is not as pronounced as in seawater.

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 wastewater 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 (Fig. 2A and 3A).

However, for those mass culture applications that require the maintenance of specific species, such as aquaculture (Goldman and 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.

5. References for Section D.

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6. Tables and Figures for Section D.

Table 1. Summary of maximum tolerable pH and average cellular chemical composition data

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

^a Minimum pH established by combination of increase in alkalinity and 1% CO₂.

^b Maximum pH for which a steady state dilution rate of 0.5 day⁻¹ could be maintained.

^c Steady state algal biomass at dilution rate of 0.5 day⁻¹.

^d Mean ± standard deviation from all pH experiments. All values were significant at the <0.01 level.

Table II. Steady state concentrations of algal biomass and constituents of $\text{CO}_2\text{-HCO}_3\text{-CO}_3^{2-}$ chemical system in cultures of S. obliquus grown at maximum pH.

Culture System	Maximum observed pH	Algal biomass (mg C · liter ⁻¹)	Alkalinity ^a (meq)	Total inorganic ^b carbon (mgC · liter ⁻¹)	Aqueous ^b CO ₂ (ng C · liter ⁻¹)
pH-Stat	10.7	552	8.3	52.2	453
Continuous Air	10.6 ^c	179	3.5	21.4	258

^a estimated from mM particulate nitrogen produced plus 2 meq HCO_3^- added initially.

^b estimated from culture pH and alkalinity.

^c average pH; range over several day period preceeding sampling was 9.4-11.2 (Fig. 4).

Figure. Legend.

- Figure 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 liter 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% CO₂ 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.
- Figure 2. Effect of culture pH on steady state concentration of algal carbon for marine continuous culture maintained at a dilution rate of $\sim 0.5 \text{ day}^{-1}$: A. Phaeodactylum tricornutum; B. Dunaliella tertiolecta. o - pH-stat control; o-continuous air.
- Figure 3. Effect of culture pH on steady state concentration of algal carbon for freshwater continuous cultures maintained at a dilution rate of $\sim 0.5 \text{ day}^{-1}$: A. Scenedesmus obliquus; B. Chlorella vulgaris. o - pH-stat control; o - continuous air.
- Figure 4. pH oscillation around steady state pH of ~ 10.6 for cultures of Scenedesmus obliquus grown with continuous air supply at dilution rate of $\sim 0.5 \text{ day}^{-1}$.
- Figure 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 tricornutum; D,H Dunaliella tertiolecta.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

D. THE EFFECT OF pH ON SPECIES COMPETITION

1. Introduction

In Section D of this study we demonstrated that the marine diatom Phaeodactylum tricornutum could tolerate alkaline pH values up to 10.3, whereas the chlorophyte Dunaliella tertiolecta was unable to grow when the pH exceeded 9.3. Although data on pH effects on marine algae are limited, there is some evidence indicating that most marine species are like D. tertiolecta and can not tolerate extreme alkaline conditions. Thus we hypothesized that in intensive cultures that are poorly buffered P. tricornutum enjoys a competitive advantage that is related primarily to pH. This hypothesis is consistent with the repeated observation that in large-scale outdoor cultures in which pH is unregulated and allowed to rise above 10 P. tricornutum often dominates (Goldman and Ryther, 1976; D'Elia et al., 1977; Goldman and Mann, 1980).

Other hypotheses to explain the success of P. tricornutum in large-scale cultures have been offered including allelopathy (Sharp et al., 1979), light limitation (Nelson et al., 1979), temperature (Goldman and Ryther, 1976), and silicon limitation (D'Elia et al., 1979; Goldman and Mann, 1980). Both temperature and silicon limitation play important roles in establishing some of the conditions favoring P. tricornutum. For example, when silicon is in excess, P. tricornutum, which does not have a silicon requirement, competes most favorably in the temperature range 10°-20°C (Goldman and Ryther, 1976); but when silicon is limiting the temperature range favoring dominance by P. tricornutum is extended down to <5°C as other cold water and silicon-requiring diatoms are eliminated from

competition (Goldman and Mann, 1980). These observations, together with the pH data from Section D lead us to suggest that P. tricornutum always will be favored when the pH is ≥ 10 and the temperature is in the range 10° - 20° C.

Because P. tricornutum is an undesired species for many mass culture applications (Goldman and Stanley, 1974), a major goal in mass culturing marine microalgae has been to find ways to prevent its incursion into and takeover of large-scale cultures. In the current study we explore further the role of pH regulation in this process by carrying out a series of competition experiments between P. tricornutum and D. tertiolecta and between two freshwater microalgae at varying pH levels.

2. Materials and Methods

a. Continuous cultures: The test algae, nutrient media, culture system, and modes of pH control were described in detail in Section D. In summary, two marine species, Phaeodactylum tricornutum (TFX-1) Bohlin and Dunaliella tertiolecta (Dun) Butcher and two freshwater species, Scenedesmus obliquus (Turp.) Kutz. and Chlorella vulgaris Beij. were grown in nutrient-enriched continuous cultures at a nominal dilution rate D of 0.5 day^{-1} . The pH in six cultures was regulated by a pH-stat system that controlled the flow of 1% CO_2 gas into the cultures to keep the pH from rising above the designated value. In the seventh culture continuous bubbling of laboratory air was provided so that the pH rose to a level controlled primarily by the photosynthetic demand for inorganic carbon. Two mM HCO_3^- was supplied in the liquid medium to allow for the initial

rise in pH to the designated value before bubbled CO_2 became the major source of inorganic carbon. In this way inorganic carbon never was limiting in the pH-controlled cultures.

b. Species competition: We cultured each species to steady state at varying pH in an overall range between 7.6 and 10.6. As described in Section D, both freshwater species grew in the pH range 7.9 to 10.6, whereas the pH ranges were 7.6 to 10.3 for P. tricornutum and 7.6 to 9.3 for D. tertiolecta. Once steady state was attained, we added the respective contaminant species ($<1\%$ of total culture biomass) to each freshwater or marine culture and observed the outcome of competition over a two to three week period. Cell counts were taken daily of the individual species in each culture using a Spencer Bright-line hemacytometer. We calculated specific growth rates μ of individual species via the following equation: $\mu = D + t^{-1} \ln (X_t \cdot X_0^{-1})$, in which t is the time interval in which a change in cell number from X_0 to X_t is observed.

3. Results

When C. vulgaris was established as the dominant species, it successfully resisted competition from S. obliquus over the entire range of pH tested (7.9 to 10.6). Similar results were obtained when S. obliquus was dominant initially and C. vulgaris was the contaminant. In all cases the contaminant species rapidly disappeared from the culture after being introduced. P. tricornutum, when dominant initially, similarly was able to

resist competition from D. tertiolecta at all pH values in the range 7.6 to 10.3, and the latter species quickly disappeared from the culture. However, when D. tertiolecta was dominant initially, the outcome of competition with P. tricornutum was a complex function of pH. Only at the lowest pH (7.60) was it impossible for P. tricornutum to grow, leaving the steady state population of D. tertiolecta unperturbed for two weeks (Fig. 1A). Then, when we increased the controller setting to pH 10, the culture pH rose only to 9.60 so that there was no further addition of CO₂-enriched air; growth of the chlorophyte stopped ($\mu = 0.05 \text{ day}^{-1}$) and cell washout ensued (Fig. 1A).

At pH values of 8.05 (Fig. 1B), 8.70 (Fig. 2A), and 9.10 (Fig. 3A) it still was possible to maintain steady state populations of D. tertiolecta although there was about a two-fold decrease in steady state cell numbers with increasing pH. Yet, in all three cultures, P. tricornutum increased exponentially after being introduced - either after a nine day lag at pH 8.05, or immediately at pH 8.70 and 9.10. Moreover, initial growth rates of the contaminant increased with increasing pH from 0.77 day^{-1} at pH 8.05 to 1.18 day^{-1} at pH 9.10 (Table II). Correspondingly, when we increased the pH from 8.70 to 9.18 (Fig. 2A) and from 9.10 to 9.40 (Fig. 3A), the growth rate of D. tertiolecta declined below the dilution rate and cell washout ensued. In both cases P. tricornutum was equal in cell number to D. tertiolecta when the experiments were terminated. Unfortunately, most of the experiments had to be terminated before the outcome of competition was complete due to difficulties in counting individual species resulting from cell clumping.

The most rapid initial growth rate of P. tricornutum (1.37 day^{-1}) was measured in the air-grown culture of D. tertiolecta initially at pH 9.3 (Fig. 2B). As P. tricornutum increased in number, the pH rose leading to a decline in the population of D. tertiolecta until it was surpassed by the diatom at the end of the experiment. Finally, when the controller was set at pH 10, the pH rose to this level only because P. tricornutum rapidly grew at 0.93 day^{-1} ; under these conditions the net growth rate of D. tertiolecta was zero, and within ten days the chlorophyte was eliminated from the culture.

4. Discussion

a. Competition at optimal pH: Based on continuous culture theory (Spicer, 1955), for any dilution rate D below the maximum specific growth rate $\hat{\mu}$, a single species culture will attain steady state. Accordingly, the impact of pH on continuous culture growth of algae (including species competition) can be described in a manner similar to the way in which the effects of other abiotic factors such as light intensity and temperature have been described. Such regulatory factors can be best quantified in terms of their modifying effects on $\hat{\mu}$ (Goldman and Carpenter, 1974). For example, each species has an optimal pH (or pH range) for growth; but above and below this optimal pH growth rates are adversely affected. Thus when D is fixed, as in the current experiments, adverse effects of pH on $\hat{\mu}$ can lead to situations where μ approaches D , with concomitant reductions in steady state biomass levels; or, in more severe cases in which $\hat{\mu}$ is diminished below D , cell washout can result.

In the current studies the experimental D of 0.5 day^{-1} was considerably below each species' $\hat{\mu}$, at least when the algae were grown at their respective optimal pH (Goldman and Peavey, 1979 and Goldman and Graham, 1981). Under these conditions the hardy weed species, which not only tolerate a broad range of pH extending into the alkaline region, but also are hardly affected by pH within that range (i.e. S. obliquus and P. tricornutum, c.f. Goldman et al., in prep), always remained dominant when exposed to a small contaminant population (initially $< 1\%$ of the total biomass). Thus pH had little effect on $\hat{\mu}$ of the dominant species so that the relationship between μ and D never was altered (i.e. $\hat{\mu} \gg D$). In an ecological sense, a key determinant of this competition is the actual ratio of dominant to contaminant species at the onset of competition. When the contaminant population is exceedingly small compared to that of the well established species the opportunities for species displacement are reduced considerably because the niche is already occupied (Whittaker et al., 1973). The same argument applies for explaining the success of both D. tertiolecta and C. vulgaris in avoiding displacement by their respective contaminants when each of these species was firmly established in culture under conditions of optimal pH. For each species μ was much greater than D and the niche was completely occupied before the contaminant was introduced.

We thus conclude that, in principal, pH control may be a simple way to maintain for sustained periods desired marine species other than P. tricornutum in large scale outdoor cultures. However, the success of this technique hinges on satisfying the requirements that the desired species

be established in sufficient numbers before contamination occurs, that the optimal pH for the desired species be well identified, and that rigid pH control be sustained throughout the culture. Moreover, the opportunities for species displacement to result from even small changes in environmental parameters other than pH (e.g. percentage CO₂ in bubbled gas, light intensity, dilution rate, nutrient flux and composition, and temperature) are well documented (Golueke, 1960; Goldman and Ryther, 1976; Goldman and Mann, 1980; Azov et al., 1981). Hence, although pH control is mandatory for preventing the displacement of a desired marine mass culture by P. tricornutum, the regulatory effects of pH control may be overshadowed by changes in other environmental parameters. Such changes could easily alter the balance in favor of P. tricornutum by lowering μ of the desired species. Then the weed diatom could compete for the niche even though the desired species initially was well-established and growing at a near-optimal pH.

b. Competition at extreme pH: In sharp contrast to the above types of subtle pH-related interactions that occur under near-optimal pH conditions, is the obvious result of competition at extreme pH between species with distinctly different pH maxima. The results of the competition experiments between the marine algae at pH values above 9.3 demonstrate this point vividly. In this case, the inability of D. tertiolecta to maintain a steady state at a dilution rate of 0.5 day⁻¹ ($\mu < D$) when the pH exceeded 9.3 (Fig. 1A and see Section D) is consistent with the outcome of the competition experiments between this alga and P. tricornutum (Fig. 3B). These findings confirm our earlier hypothesis (Goldman et al., in

prep.) that, because most marine algae are like D. tertiolecta in not being able to grow at high pH, P. tricornutum can grow unrestricted in unbuffered cultures when the pH rises to 10 and above. Hence, the demonstrated success of P. tricornutum in previous large-scale outdoor experiments (c.f. Goldman and Ryther; D'Elia et al., 1977; Goldman and Mann, 1980) more than likely was the result of unregulated pH leading to alkaline conditions.

c. Competition under pH stress: Unlike P. tricornutum and S. obliquus which are only slightly affected by alkaline pH, both C. vulgaris and D. tertiolecta are extremely sensitive to increasing pH above the respective optimal levels (Goldman et al., in prep). On this basis, intuitively it would appear that only under optimal pH could these pH-sensitive species could compete favorably with species like P. tricornutum and S. obliquus. Yet, the results of the competition experiments performed under non optimal pH conditions (Figs. 1-3) are not so easily interpreted.

On the one hand, the increasing success of P. tricornutum in invading stable cultures of D. tertiolecta when the pH was raised from 8.05 to 9.10 (as demonstrated by the increasing initial growth rates of the diatom when first introduced into the cultures - Table I), appears coupled to the pH-dependent decrease in steady state biomass of D. tertiolecta (Fig. 1 in Goldman et al., in prep). Under such conditions it is most probable that μ of the chlorophyte decreased dramatically with increasing pH (reflecting the pH stress), thereby easing the pressure on P. tricornutum to compete

for the available resources. This argument is consistent with the observation that during the initial periods when P. tricornutum successfully encroached the cultures, steady state of D. tertiolecta was sustained for periods up to three weeks (Figs. 1B, 2A, and 3A).

Our inability to maintain the cultures long enough to observe definitively the outcome of competition between the two species precludes any conclusions as to whether or not precise pH control (7.60 as opposed to 8.05) is necessary to prevent takeover by a contaminant such as P. tricornutum under seemingly optimal growth conditions of a species like D. tertiolecta. However, that P. tricornutum could even gain a foothold at $\text{pH} \geq 8.05$, but not at $\text{pH} 7.60$ is suggested that the diatom eventually would have displaced D. tertiolecta at any $\text{pH} \geq 8.05$.

In contrast, C. vulgaris, although displaying the same sensitivity to high pH as the marine chlorophyte, was able to resist competition from the pH-insensitive S. obliquus at any pH between 7.9 and 10.3 as long as it was dominant initially. Such a response is suggestive of an allelopathic interaction between the two species. In fact, C. vulgaris, along with many other algal species, can excrete compounds toxic to other algae under certain conditions, one of which is high pH (Pratt and Fong, 1940, Rice, 1954, Proctor, 1956, Kroes, 1971; Lam and Silvester, 1979; Chan et al., 1980). Proctor (1957) identified these products as long chain fatty acids which become more soluble (and possibly more toxic) with increasing pH. Thus the ability of C. vulgaris to persist at all pH values when dominant initially may reflect, on the one hand, an ability to occupy a niche through excellent growth at optimal pH, and, on the other hand, an

ability to survive under pH stress by eliminating competitors via allelopathetic interactions. Once again, the relative ratio of initially dominant to contaminant populations probably is a major determinant of the outcome of this form of competition.

Sharp et al. (1979) suggested that the dominance of P. tricornutum when in competition with the diatom Thalassiosira pseudonana (3H) also resulted from allelopathy. They based this conclusion on results demonstrating that when stationary phase cells of P. tricornutum were added to steady state continuous cultures of T. pseudonana (3H) both species washed out. However, D'Elia et al. (1979) could find no evidence for allelopathy in competition experiments between these diatoms in batch culture. The latter authors subsequently found that P. tricornutum grows faster than T. pseudonana (3H) at very low light intensity, leading them to postulate that light reduction via self shading among cells in mass culture could provide P. tricornutum with a competitive edge (Nelson et al., 1979).

Our results do not support either of the above explanations for the success of P. tricornutum. First, if low average light intensity is a prerequisite for dominance by P. tricornutum, then invasion by the diatom should have been most pronounced at low pH where the biomass of D. tertiolecta, (and concomitantly, shelf shading) was maximum, and least effective at high pH (9.10) where opposite conditions prevailed. Although the increase in the initial growth rates of P. tricornutum (Table I) may have been due to more available light as the steady state biomass of D. tertiolecta decreased with increasing pH, the results shown in Figures 1-3 are not consistent with an enhancement effect favoring P. tricornutum at low culture light intensities.

Similarly, it is hard to reconcile the ability of P. tricornutum to invade and establish a firm foothold in the D. tertiolecta cultures to an allelopathic interaction when there was no measurable deviation from steady state growth of the chlorophyte during the early stages of successful invasion by P. tricornutum. As mentioned earlier, the success of P. tricornutum under these conditions most probably was related to an increase in the available resources (nutrients, light, etc) as the culture of D. tertiolecta was increasingly stressed when the pH was elevated.

The role of pH in the competition studies of Sharp et al. (1979) and Nelson et al. (1979) needs further elucidation because pH was neither regulated nor monitored in those experiments; and it is well established that T. pseudonana (3H), like D. tertiolecta, can not grow at pH values above 9-9.5 (Goldman, 1976; Pruder and Bolton, 1979).

5. Conclusions

In natural aquatic ecosystems competition among phytoplankton species is extraordinarily complex, involving the simultaneous and compound impacts of numerous factors. However, in large scale mass cultures many of the important regulating factors such as nutrient limitation, grazing pressure, cell sinking, etc. are eliminated. Controls on competitive interactions thereby are reduced to a relatively few factors such as light intensity, temperature and chemical constraints. Culture pH clearly is one of the important chemical factors influencing the outcome of competition in intensive cultures. Without pH control, pH increases are regulated solely by the photosynthetic demands of the algae in relation to the

available aqueous inorganic carbon sources (Goldman et al., 1972). On this basis, the species that is least affected by high pH will dominate eventually. Consequently, P. tricornutum dominates intensive marine cultures because of its unique ability to not only tolerate high pH, but to flourish under such conditions. Other suggested reasons for this dominance, such as light responses and allelopathy, can only be considered as secondary effects, if they are important at all.

It may be possible to maintain other more desired species in intensive culture with pH control; but the attainment of such conditions may be difficult because P. tricornutum grows well over a wide pH range and seems to be capable of exploiting rapidly any competitive situation in which even slight stress is put on the already established and desired species. However, such questions will only be answered definitively when long-term outdoor experiments involving pH manipulation are performed.

6. References for Section E.

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7. Tables and Figures for Section E.

Table I. Initial growth rates of *P. tricornutum* when introduced as contaminant in steady state cultures of *D. tertiolecta*.

Culture pH	CO ₂	Dilution Rate (day ⁻¹)	Initial Growth Rates (day ⁻¹)
7.60	1	0.44	0
8.05	1	0.59	0.77 ^a
8.70	1	0.59	0.90
9.10	1	0.60	1.18
9.50	0.036	0.55	1.37
10.05	None	0.48	0.93

^a After 9 day lag.

Figure Legend.

- Figure 1. Competition between Dunaliella tertiolecta and Phaeodactylum tricornutum in continuous culture maintained at a nominal dilution rate of 0.5 day^{-1} and at varying pH. Except where noted, pH control was with $\% \text{ CO}_2$, regulated with a pH-stat system. Dunaliella tertiolecta (o) initially was at steady state and P. tricornutum (o) was introduced as the contaminant. Symbols and legend are the same for all figures. Dashed lines were drawn by eye to estimate growth rates of P. tricornutum and washout rates of D. tertiolecta. A, pH 7.60 and 9.60; B, pH 8.05.
- Figure 2. Competition between Dunaliella tertiolecta and Phaeodactylum tricornutum in continuous culture maintained at a nominal dilution rate of 0.5 day^{-1} and at varying pH. A, pH 8.70 and 9.18; B, pH 9.50 and 9.70 (0.036 CO_2 in air supplied continuously).
- Figure 3. Competition between Dunaliella tertiolecta and Phaeodactylum tricornutum in continuous culture maintained at a nominal dilution rate of 0.5 day^{-1} and at varying pH. A, pH 9.10 and 9.40; B, pH 9.45 and 10.05

Figure 1

Figure 2

Figure 3