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STUDIES ON THE USE OF RADIOSULFATE TO DETERMINE THE PHYSIOLOGICAL STATE, GROWTH RATE AND RATE OF PROTEIN SYNTHESIS BY MARINE PHYTOPLANKTON

by

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Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at Dalhousie University

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Approved by:

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ABSTRACT

A technique using $^{35}SO_4^{2-}$ was developed to assess the physiological state, with respect to division rate and rate of protein synthesis, of marine phytoplankton. Radiosulfur is incorporated predominantly into protein and was about 50% of total sulfur uptake for Thalassiosira weissflogii (=fluviatilis). Rates of protein synthesis calculated from the rate of sulfur incorporation (S inc) and a protein:sulfur ratio of 36:1 (w/w) agreed with measured rates during active cell growth but generally exceeded those measured during the stationary phase.

Significant positive correlations were found between S inc and changes in cell number, in vivo fluorescence, chlorophyll a (chl a) and protein concentration in batch culture. S inc per cell declined as T. weissflogii progressed from exponential phase to late senescence. Cultures of Amphidinium carteri, Dunaliella tertiolecta and Pavlova (=Monochrysis) lutheri behaved similarly. Changes in S inc detected the onset of the stationary phase 24 h before it was observed by measuring ^{14}C uptake or cell numbers.

The ratio of S inc in the dark to that in the light was low and relatively constant during exponential growth of T. weissflogii and became elevated during the stationary phase. The ratio of ^{14}C uptake rate to S inc in the light followed a similar pattern. Cultures grown in a N-limited chemostat gave ratios similar to those observed for cells in exponential growth. Relationships were found between division rate and ratios of dark:light S inc, carbon uptake rate : S inc, and S inc : chl a.

The technique was applied to populations from coastal waters of Nova Scotia, Peru and the Davis Strait. Ratios of sulfur uptake rate to chl a measured in the Davis Strait indicated the direction of development of a phytoplankton bloom as confirmed by temporal changes in chl a concentration.

ABBREVIATIONS

ATP	Adenosine triphosphate
CCCP	Carbonylcyanide 3-chlorophenylhydrazone
chl *a*	Chlorophyll *a*
C up	Rate of photosynthetic carbon uptake
DCMU	3-(3,4-dichlorophenyl)-1, 1-dimethylurea
d.p.m.	Disintegrations per minute
E	Einsteins
K_m	Michaelis-Menten half saturation constant
POC	Particulate organic carbon
S inc	Rate of sulfur incorporation
S up	Rate of sulfur uptake
TCA	Trichloroacetic acid
μ	Specific growth rate (divisions d^{-1})
V_{max}	Maximum rate of nutrient uptake

CONVENTION

In Figures which show an exponent in the scale, multiply the digit on the axis by the power of 10 in the scale to determine the true value of the number.

Example: (Fig. 5) For "2.0" on the y axis, and "(cells ml^{-1}) X 10^4" on the scale for the y axis, the true value of the number is 20,000 cells ml^{-1}.

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INTRODUCTION

Although there is a large literature on *in situ* rates of phytoplankton photosynthesis, there has been little work done to assess the physiological state and to measure the growth rate *per se* of marine phytoplankton in the field (Eppley and Strickland, 1968; Healey, 1973). Physiological state is an ambiguous term, often used interchangeably with physiological age, physiological vigour, nutritional status or growth status (Platt and Subba Rao, 1970; 1975; Healey, 1975). It therefore seems desirable to define terms. Physiological state is a manifestation of the interaction between the organism and its nutritional, chemical and physical environment. It reflects the phytoplankter's capacity to divide under a given environmental condition. Thus, cells in a poor physiological state divide slowly due to alterations in enzyme levels or activities and accompanying changes in the pattern of metabolism in response to the environment (Schimke, 1973; Goldberg and Dice, 1974).

Even though cell growth does not necessarily accompany cell division (Shrift, 1959; Eppley and Strickland, 1968), I will adopt the premise stated by Nierlich (1978) that "growth is considered primarily a manifestation of the ability of the cell to make protein". This is justified because protein is the constituent of enzymes and comprises a large percentage of the cell's biomass.

With a knowledge of the physiological state of phytoplankton, one can determine the growth potential of a population, and hence its availability to herbivores. The generation time of phytoplankton is often chosen by modelers as the scaling factor to which all other biological processes in the marine ecosystem can be related (Platt and Denman, 1975; Wroblewski and O'Brien, 1976). Reliable measurement of division rates would therefore have important theoretical implications in refining models of marine systems. Information on the physiological state of phytoplankton, coupled with measurements of rates of cell division, grazing, sinking and advection would provide greater insight into the dynamics of plankton ecosystems. We are still far from being able to accurately determine all of these factors in the ocean. The present study is an attempt to approach the problem by providing the foundation for a technique to assess the physiological state of marine phytoplankton by measuring the incorporation rate of ^{35}S, provided as $^{35}SO_4^{2-}$, into the cellular protein fraction.

The physiological state of phytoplankton can be assessed by the measurement of chemical composition or of the rates of selected metabolic processes. This study takes the second approach, using ^{35}S as a tag for measuring the synthesis of cellular protein. It is not intended that the ^{35}S

incorporation technique replace existing methods for assessing the growth status of phytoplankton, only that it provide an alternative way to examine the problem.

The Use of Chemical Composition to Assess Growth Status.

Physiological state. Most methods used to study the physiological state of phytoplankton have relied on measurements of chemical composition (Healey, 1973; 1975; 1978). Laboratory studies have compared the bulk chemical composition of species grown under nutrient-sufficient (Parsons et al., 1961; Ricketts, 1966; Myklestad, 1975; Conover, 1975a; Haug and Mykelstad, 1976), or nutrient-deficient conditions (Myklestad and Haug, 1972; Sakshaug et al., 1973; Paugh, 1975; Skoglund and Jensen, 1976; Sakshaug and Holm-Hansen, 1977, Myklestad, 1977; Rhee, 1978). The culture of phytoplankton in dialysis sacs suspended in situ has been used to obtain cells with a physiological state close to that of the same species growing in the natural environment (Sakshaug, 1977; Kossut and Maestrini, 1977, Sakshaug and Jensen, 1978). The chemical composition of natural assemblages of phytoplankton has been studied by enclosing the population in large-volume containers (McAllister et al., 1961; Antia et al., 1969; Strickland et al., 1969), or by in situ studies in fjords (Haug et al., 1973; Sakshaug and Myklestad, 1973; Jensen and Sakshaug, 1973), basins (Conover, 1975b), bays (Platt and Subba Rao,

1970; Platt and Irwin, 1973), offshore coastal areas (Eppley et al., 1971, 1977) or less frequently, in the open ocean (Perry, 1976). These researchers showed that phytoplankton have similar chemical compositions under optimal growth conditions, and exhibit similar changes in composition as a result of nutrient deficiencies.

Several laboratory studies have expressed chemical composition as ratios of cellular parameters which have then been related to the phytoplankter's physiological state (Healey, 1975; 1978). The C:N ratio (atomic) generally increases from about 6.0 for cells growing exponentially up to about 35 during nitrogen-deficiency (Sakshaug and Holm-Hansen, 1977). The C:chl a ratio (w/w) varies from about 30 in exponentially growing cells to over 100 during nitrogen-deficiency (Strickland, 1960; Sakshaug and Holm-Hansen, 1977) and is also influenced by light intensity. The C:ATP ratio (w/w) is about 250 for exponentially growing cells (Holm-Hansen, 1970) but may vary from 95 during nitrogen deficiency to about 1800 during phosphorus deficiency (Sakshaug and Holm-Hansen, 1977). The lowest ratios of C:chl a and C:N reported by McAllister et al. (1961) and Antia et al. (1963) for a mixed natural algal population enclosed in a large-volume plastic sphere, are lower than those of the above laboratory studies.

Except in virtually monospecific bloom conditions (e.g., Platt and Subba Rao, 1970; Sakshaug and Myklestad, 1973; Conover, 1975b), the determination of chemical composition is not suitable for application to natural populations. Chemical analyses are often tedious, and results are obscured by differences in species composition (Sakshaug et al., 1973; Haug et al., 1973), and interference by microzooplankton (Mayzaud and Martin, 1975) or detritus (McAllister et al., 1961). Although laborious to measure, the adenylate energy charge may be a useful index of physiological state with respect to nutrient starvation (Falkowski, 1977).

Division rate. If it is assumed that the rate of cell division is related to the growth potential of a phytoplankton population, then division rate is a reflection of physiological state. Many methods are available for measuring phytoplankton division rates (Eppley, 1972), and this perhaps demonstrates the inadequacies of any single approach. Growth may be calculated directly from changes in cell numbers or cellular constituents with time, an approach usually better suited for laboratory cultures rather than field situations because of contamination by detritus.

Researchers have circumvented the problem of detrital interference by measuring increases in living particulate material with a Coulter Counter (Sutcliffe et al., 1970;

Sheldon et al., 1973), or by ATP analysis (Sheldon and Sutcliffe, 1978). These workers obtained generation times of about 3 h for microplankton, predominantly phytoplankton, of the Sargasso Sea. This is more than an order of magnitude faster than others have found for phytoplankton assemblages of the oligotrophic central oceanic waters using ^{14}C incubation methods (Eppley et al., 1973). Alternative approaches for measuring generation times are clearly needed to help resolve these differences.

Plant pigments are commonly used as an index of living phytoplankton biomass. Eppley and Sloan (1966) developed an equation for calculating the growth rate of phytoplankton in culture from chlorophyll a content, light intensity and temperature data. The value of their equation lies in the information gained about growth regulating factors rather than in its applicability to natural phytoplankton assemblages. Problems may arise from the incomplete extraction of pigments and from uncertainties concerning the best equations for use for their determination (Humphrey, 1961). Moreover, it is often not possible to distinguish between changes in the concentration of pigments due to algal growth or to the physiological effects of light intensity, nutrient status or cell age (Hobson and Pariser, 1971; Healey, 1973; Riper et al., in press).

The division rate of phytoplankton growing at steady-state in a chemostat is often believed to be a hyperbolic function of its chemical composition (e.g., Droop, 1973). Chemostat studies have compared ratios of cellular parameters in the hope that growth rates at sea may be estimated from such ratios. The chl:C ratio vs. growth rate relationships of Thomas and Dodson (1972), Caperon and Meyer (1972), Eppley and Renger (1974), and Picard (1976) differ considerably. The N:C vs. growth rate curves of Thomas and Dodson (1972), Caperon and Meyer (1972), Eppley and Renger (1974), Malone et al. (1975) and Picard (1976) show greater similarity, especially when the measured division rate is normalized by the maximum attainable division rate (Donaghay et al.,1978). For elements such as N, C and Si which constitute a large percentage of the cell weight, the slope of the elemental ratio vs. division rate curve may approach infinity, thus diminishing the usefulness of the relationship (Goldman and McCarthy, 1978). Moreover, differences in species composition and interference by detritus complicate the interpretation of such ratios in natural communities (Slawyk et al., 1978). Measurement of an N:C uptake ratio eliminates the problem of detrital interference, but this ratio is equivalent to the N:C composition ratio only at steady-state (Eppley and Renger, 1974; Slawyk et al., 1978).

The Use of Metabolic Rate to Assess Growth Status

Physiological state. Enzymatic and photosynthetic rate measurements have provided some information on the physiological state of marine phytoplankton. Enzymes studied include: nitrate reductase (e.g., Eppley et al., 1969), glutamate dehydrogenase (e.g., McCarthy and Eppley, 1972), alkaline phosphatase (e.g., Kuenzler and Perras, 1965), ribulose bisphosphate carboxylase (e.g., Mukerji and Morris, 1976), and activity of the electron transport system (e.g., Packard, 1971). Although these methods give an instantaneous measurement without the problems introduced by containing and incubating a sample, they show the potential rather than actual metabolic rate. Fluorescence induced by DCMU, an inhibitor of photosynthetic electron transport activity, may provide an alternative method for assessing physiological state if properly understood and employed (c.f., Samuelsson and Öquist, 1977).

The advent of the ^{14}C method for measuring primary production (Steemann Nielsen, 1952) has resulted in a proliferation of data. The method is widely used despite uncertainties concerning dark uptake of ^{14}C (Morris et al., 1971a), respiration in the dark (Eppley and Sharp, 1975), recycling of respired ^{14}C (Steemann Nielsen, 1955), photorespiration (Tolbert, 1974), and excretion of ^{14}C-

labelled organic compounds (Sharp, 1977). Because it is so easily and routinely measured, however, ^{14}C uptake has been used to assess the growth status of phytoplankton. The assimilation ratio (photosynthetic rate per unit chlorophyll a at light saturation) provides an index of general nutrient deficiency (Curl and Small, 1965; Thomas and Dodson, 1972). However, the method is not standardized, and physiological arguments may be leveled against its use. Physiological state is a function of both the activity of photosynthetic enzymes and the concentration of chlorophyll, (c.f., Steemann Nielsen, 1974) but measurement of photosynthesis at light saturation ignores the effect of the latter. Moreover, expressing photosynthetic rate on a unit chlorophyll basis detracts from the ratio's sensitivity, as both parameters decrease with increasing nutrient deficiency. Finally, temperature may have a greater effect on the assimilation ratio than would nutrient deficiency (Eppley, 1972).

The change in photosynthetic rate due to selective nutrient enrichment has been used to determine the degree of nutrient deficiency and to pin-point the probable limiting nutrient (Thomas, 1969; Glooschenko and Curl, 1971). However, the photosynthetic response may be complicated by competition between nutrient uptake and carbon fixation systems (Falkowski and Stone, 1975), the initial

degree of nutrient deficiency, the form in which the nutrient is given, the light intensity used, and by the time lag between addition of nutrients and the photosynthetic response. These factors, combined with the possibility that photosynthetic rate or growth rate may not be nutrient-limited in the ocean (c.f., Steemann Nielsen, 1978; McCarthy and Goldman, 1979; Goldman et al., 1979), may be responsible for some of the contradictory results in the literature concerning the effects of addition of nutrients on the assimilation ratio (Healey, 1973; Fogg, 1975). Nutrient deficiency has also been identified by the enhancement of ^{14}C fixation in the dark due to the addition of ammonium (Morris et al., 1971b; Yentsch et al., 1977; Yentsch, 1977).

Division rate. Specific growth rates can be calculated from measurements of ^{15}N or ^{14}C uptake. Dugdale and Goering (1967) proposed that the rate of ^{15}N uptake can be considered a specific growth rate in terms of nitrogen with units of time^{-1}, if expressed as mass of nitrogen taken up (mass of particulate nitrogen)$^{-1}$ (time)$^{-1}$. McCarthy and Eppley (1972) verified that division rates calculated from rates of ^{15}N uptake for diatoms growing in an enriched seawater sample agreed with estimates based on cell counts. Unfortunately, there are drawbacks to the use of ^{15}N for division rate determinations under natural conditions. The form in which ^{15}N

is supplied (i.e., either as nitrate, nitrite, ammonium or urea) is not necessarily representative of all the possible nitrogen compounds available to the phytoplankter in the water column. This is analogous to, although perhaps not as serious as, the problem of measuring heterotrophic potential by supplying a single ^{14}C-labelled substrate (c.f., Monheimer, 1974a). Detrital particulate nitrogen dilutes the living fraction resulting in an underestimate of the ^{15}N uptake rate (Dugdale and Goering, 1967) and hence division rate. Addition of tracer nitrogen, even at a mass equivalent to 10% of that present in seawater, may perturb the nutritional state of the system and result in the measurement of artificially high rates of nitrogen uptake (Dugdale and Goering, 1967). This problem becomes particularly significant when the ambient nitrogen concentration approaches the limits of analytical detection, because the amount of nutrient enrichment due to the addition of tracer must then be estimated.

The rate of nutrient uptake is equivalent to division rate only at steady-state when the nutrient taken up is not pooled in vacuoles but is channeled directly into growth (Eppley and Thomas, 1969; Caperon and Meyer, 1972). Steady-state, as defined by microbiologists (Jannasch, 1974), may rarely be established

in natural marine environments (McCarthy and Goldman, 1979). Finally, the ^{15}N method may not be sensitive enough to determine protein synthesis by measuring the accumulation of ^{15}N in the protein fraction.

Specific growth rates in the field are commonly calculated from the rate of ^{14}C uptake per unit carbon biomass (Eppley, 1972). While much information about phytoplankton growth has been gained by this method, it has two disadvantages. First, it is difficult to determine biomass in terms of carbon. Direct determination of carbon suffers from detrital interference and difficulties in methodology. Indirect carbon determination relies on empirical equations to convert cell volume to carbon (Strathmann, 1967) or on ratios of C:chl (Eppley, 1968) or C:ATP (Holm-Hansen, 1970). The ratios change markedly, however, depending on the phytoplankter's physiological state (Sakshaug and Holm-Hansen, 1977) and on the method for determining the C:chl ratio of natural phytoplankton (Banse, 1977). The specific growth rate calculated will therefore vary greatly according to the ratio chosen (c.f., Eppley, 1972). Secondly, carbon is found in all organic compounds synthesized by the organism. Some compounds, however, will be synthesized more rapidly than others depending on the phytoplankter's physiological state. Protein synthesis accompanies active growth, while lipid and carbohydrate synthesis predominate

during senescence (Antia et al., 1963; Strickland et al., 1969; Hobson and Pariser, 1971; Myklestad and Haug, 1972; Conover, 1975a; Lehman, 1976; Myklestad, 1977). Lipids and carbohydrates accumulate as sinks for excess photosynthate formed when the cell is exposed to light energy and carbonate in the absence of nitrogen or phosphorus (Healey, 1973; Lehman, 1976). The ^{14}C method therefore may not give an accurate representation of division rate if the phytoplankton population is nutrient stressed. The degree to which phytoplankton are nutrient limited in the world's oceans remains an open question (Steemann Nielsen, 1978; McCarthy and Goldman, 1979; Goldman et al., 1979).

More precise information on physiological state is derived by measuring the rate of synthesis of individual ^{14}C-labelled compounds. Olive and Morrison (1967) and Olive et al. (1969) found that more ^{14}C entered the protein than the carbohydrate fraction in rapidly growing natural populations of freshwater phytoplankton. Morris et al. (1974) and Morris and Skea (1978), on the other hand, found increased incorporation of ^{14}C into protein in nitrogen-deficient marine algae, a paradox that was not adequately resolved. The presence of phytoplankters exhibiting elevated phosphoenolpyruvate carboxylase activity during the stationary phase of growth may lead

to the rapid synthesis of C_4 compounds and an increased proportion of ^{14}C entering protein (Mukerji et al., 1978).

While these studies increased our understanding of the physiological state of natural populations, they suffer because of the long fractionation procedures involved and the fact that carbon occurs in all cellular organic matter. The ^{35}S incorporation technique takes advantage of the fact that sulfur is found in relatively few compounds. Of those in which it is located, protein should be an excellent indicator of physiological state.

Literature Review on Sulfate Reduction by Algae.

In the biological sulfur cycle dissimilatory sulfate reduction, with the production of hydrogen sulfide, is carried out on a large scale predominantly by obligately anaerobic bacteria of the genus Desulfovibrio (Postgate, 1968; Trüper, 1978). Assimilatory sulfate reduction, resulting in the production of organic compounds containing reduced sulfur, is carried out by bacteria, blue-green algae, eukaryotic algae, fungi and higher plants (Schiff and Hodson, 1973).

Several studies indicate a special requirement for sulfur in algal cell division (Shrift, 1959; Hase et al., 1958; 1959; Cook and Hess, 1964). Sulfate is taken up by active transport (Wedding and Black, 1960; Deane and O'Brien, 1975; Utkilen et al., 1976; Jeanjean and Broda, 1977; Coughlan, 1977), reduced to the thiol level found in

the sulfur-amino acids cysteine and methionine, and then built into protein (reviewed by Schiff, 1962; Thompson, 1967; Postgate, 1968; Roy and Trudinger, 1970; Schiff and Hodson, 1970; 1973; Wilson and Reuveny, 1976; Schwenn and Trebst, 1976; Anderson, 1978). Protein synthesis, in turn, is required for continued cell division. A measure of the rate of ^{35}S incorporation into protein, therefore, should indicate the cell's ability to grow under a given environmental condition.

The use of radiosulfate to assess the growth status of phytoplankton has several advantages over existing approaches:

1) Addition of radiosulfate to the incubation bottles should not perturb the nutritional state of the system, in contrast to studies of nitrogen (Dugdale and Goering, 1967) and phosphorus limitation. This is because sulfate, the second most abundant anion in seawater at 25-30 mmol l^{-1} (Culkin, 1965), is not likely to be a growth-limiting nutrient. Radiosulfate uptake by marine phytoplankton is nevertheless measurable in the presence of this high ambient sulfate background. It is expected that phytoplankton would utilize sulfate in response to their physiological needs rather than on the availability of the anion.

2) Sulfur is located in relatively few cellular compounds. Most of it is found in cysteine, methionine and protein (c.f., Datko et al., 1978) while ionic sulfate may be

pooled in vacuoles. Sulfur-containing lipids are characteristic of all photosynthetic organisms (Collier and Kennedy, 1963). However, except for unusual cases (Haines, 1965) most algae contain relatively little sulfolipid. Sulfated polysaccharides, although common in some macroalgae and a few unicellular species (Ramus and Groves, 1972; Evans et al., 1974) are insignificant in marine phytoplankton. The remainder of cellular sulfur is distributed among intermediary metabolites (e.g., adenosine phosphosulfate, glutathione) and other minor constituents (e.g. coenzyme A, biotin, thiamine, ferredoxin). Most of the sulfur compounds other than ^{35}S-protein can be eliminated by treatment of the cells with TCA and methanol:ether. The method thus becomes relatively specific as a marker for cellular protein. Sulfur-35 has been used to determine rates of protein synthesis by bacteria growing in the rumen of animals (e.g., Beever et al., 1974; Lundri and Arora, 1976).

3) The basic metabolism of sulfate has already been elucidated, at least for several species of freshwater algae (e.g., Wilson and Reuveny, 1976; Tsang and Schiff, 1978). Important theoretical considerations and methods for measuring ^{35}S in cellular fractions are discussed by Roberts et al. (1955).

4) Sulfate is not metabolized by protozoa or higher animals which must obtain their sulfur requirements by

ingesting plant material (Schiff and Hodson, 1973). Bacteria reduce sulfate, but most should be eliminated by using 1.0 µm Nuclepore filters (c.f., Berman, 1975; Azam and Hodson, 1977; Salonen, 1974) or by gentamycin, an inhibitor of bacterial protein synthesis (Caskey, 1973).

5) The methodology for measuring ^{35}S incorporation is similar to that for measuring primary production with ^{14}C. Many of the technical problems have therefore been discussed and at least partially resolved in the literature for ^{14}C. Retention of radioactivity by filters appears to be more serious for ^{35}S (Jordan et al., 1978; M. Jordan, pers. comm.; Campbell and Baker, 1978a) than for ^{14}C (Morris et al., 1971a; Nalewajko and Lean, 1972; Williams et al., 1972; McMahon, 1973). This problem was encountered and independently resolved in the present study.

6) Sulfur-35 is a weak beta-emitting isotope of energy (0.167 MeV) similar to that of ^{14}C (0.156 MeV) thus ^{35}S can be measured easily with a scintillation counter using discriminators set for ^{14}C. The hazards of working with ^{35}S are comparable with those of ^{14}C. The half-life of ^{35}S (87.2 d) is an advantage in case of the need for decontamination procedures, while still of useful duration for work on cruises.

Virtually all of the previous ecological work using radiosulfate has been done in freshwater systems. The only known study of a marine phytoplankter used an artificial

seawater medium with all sulfate salts replaced by chloride equivalents (Deane and O'Brien, 1975).

Kylin (1964a; 1964b; 1966) studied the uptake of radiosulfate by Scenedesmus in relation to phosphate limitation. Monheimer (1972; 1974a; 1975a; 1975b) introduced a method for estimating photosynthetic plus heterotrophic production of microplankton (algae plus bacteria) in freshwater lakes by measuring the uptake rate of radiosulfate. Production by freshwater bacteria was measured by dark incubation with radiosulfate, with the assumption that algae do not take up sulfate in the dark (Monheimer, 1974b; 1975c; Jassby, 1975; Campbell and Baker, 1978a; 1978b). However, it has been shown that algae do take up sulfate in the dark, although at a reduced rate (Wedding and Black, 1960; Miyachi and Miyachi, 1966; Vallée and Jeanjean, 1968; Deane and O'Brien, 1975; Jeanjean and Broda, 1977; Coughlan, 1977; Monheimer, 1978; Campbell and Baker, 1978a; 1978b; P. Campbell, pers. comm.). These studies assumed a constant C:S ratio to convert sulfur uptake to carbon production. Jassby (1975) and Campbell and Baker (1978a) used a C:S weight ratio of 50:1 based on the sulfur content of E. coli reported by Roberts et al. (1955), while Monheimer (1972; 1974a; 1975a) applied a ratio of 500:1 based on the average C:S weight ratio for "pure organic materials" (Stuvier, 1967). However, Jordan and Peterson (1978) found that the

C:S ratio varied depending on the physiological state of the organism. The weight ratios for bacteria in batch culture were highest in the early exponential growth phase and declined in stationary phase, with a mean of 103:1 for all samples (Jordan and Peterson, 1978). The C:S uptake ratios (w/w) for three species of freshwater chlorophytes in batch culture varied from 150:1 to 10,000:1 (Monheimer, 1978). I have tested the hypotheis that the variation in C:S uptake ratio may be used to determine the physiological state of marine phytoplankton.

Objectives of the Study

The objective of this study was to establish the foundation for a technique to assess the physiological state of marine phytoplankton in the field by measuring the incorporation rate of ^{35}S. The technique was developed primarily in the laboratory where each step could be tested under controlled conditions. Investigation was mainly limited to the diatom Thalassiosira weissflogii, with preliminary examinations of Dunaliella tertiolecta, Amphidinium carteri and Pavlova (=Monochrysis) lutheri. Specific objectives were to:

1) Establish that the uptake of radiosulfate by phytoplankton, as represented by the above species, could be measured in the presence of the high ambient sulfate concentration of seawater.

2) Overcome technical problems associated with filtering ^{35}S-labelled phytoplankton from seawater containing added radiosulfate,

3) Adopt a method for obtaining the ^{35}S-labelled protein fraction, and to determine if the measured radioactivity was associated predominately with that fraction,

4) Determine the characteristics of ^{35}S incorporation during the exponential growth and stationary phases in batch culture and also in nitrate-limited chemostat culture,

5) Look for correlations between the rate of ^{35}S incorporation and the rate of ^{14}C uptake, changes in cell number, protein, chlorophyll *a* and ATP concentrations during cell growth,

6) Determine the possibility of using ^{35}S to measure division rates and rates of protein synthesis,

7) Establish indices which could be used to relate ^{35}S incorporation in the field to ^{35}S incorporation data obtained under controlled conditions in the laboratory,

8) Develop a method to differentiate between the uptake of radiosulfate by phytoplankton and by bacteria, and

9) Field-test the ^{35}S incorporation technique using natural assemblages of phytoplankton in several environments.

MATERIALS AND METHODS

Culturing Procedures

Organisms used were Thalassiosira weissflogii (Grunow) G. Fryxell and Hasle, comb. nov. (=Thalassiosira fluviatilis Hustedt), Bacillariophyceae (Guillard's strain), Amphidinium carteri Hulburt, Dinophyceae (Guillard's strain), Pavlova (=Monochrysis) lutheri (Droop) Green, Chrysophyceae, and Dunaliella tertiolecta Butcher, Chlorophyceae (Guillard's strain). Cultures were kindly provided by Dr. J. S. Craigie from the Atlantic Regional Laboratory collection, National Research Council of Canada.

Organisms were maintained in axenic culture in f/2 medium (Guillard and Ryther, 1962) made with natural filtered seawater from the North West Arm of Halifax Harbour. The medium contained ambient seawater concentrations of ionic sulfate, and no attempt was made to replace sulfate salts with chloride equivalents in the trace metal mix.

Stock cultures were maintained and experiments were carried out in a constant temperature incubator (Environator Corp., West Des Moines, Iowa) at 20°C. Illumination, provided by cool white fluorescent lamps with a 16:$\overline{8}$ h or 12:$\overline{12}$ h photoperiod as stated, was about 100 $\mu E\ m^{-2}\ s^{-1}$ at the flask surface as measured by a quantum sensor (LI-185 Quantum/Radiometer/Photometer, Lamda Instruments, Lincoln, Nebraska).

Batch culture system. Batch culture experiments were carried out under the above light and temperature conditions with nutrients supplied as stated. Cultures were stirred with a Teflon-coated magnet and by bubbling with air passed through activated charcoal, distilled water and sterile cotton.

Chemostat culture system. The culture vessel was a 2 ℓ Pyrex reagent bottle filled to 800 ml with f/2 medium containing 18 µM nitrate. Medium was metered from a 16 l reservoir through silicone tubing by a Polystat peristaltic pump (Buchler Instruments, Fort Lee, New Jersey). Culture conditions were as above. Air inflow forced excess medium through an overflow tube to maintain a constant culture volume. Samples were withdrawn by closing the overflow tube, thus forcing medium through an exit siphon. The system was run as a batch culture until late exponential growth phase (3-4 d) after which the chemostat was run at a 50% turnover rate d^{-1} (= 16.8 ml h^{-1} flow). Steady-state, defined as less than a 10% change in cell concentration (c.f., Jannasch, 1974) was generally achieved after 4 to 5 d.

Analytical Procedures

All analyses were carried out in triplicate. Cellular protein was analysed according to Dorsey et al. (1977; 1978) using bovine serum albumen as a standard. Five to 10 ml of

sample were collected on a 25 mm diameter Type A-E glass fiber filter (Gelman Instrument Co., Ann Arbor, Michigan) and frozen at -20°C until analysed, usually no longer than 4 d. Membrane filters are unsuitable as they interfere in the assay, giving a dark purple colour (T. Dorsey, pers. comm.).

Kjeldal digestion of organic nitrogen was carried out according to Strickland and Parsons (1972), and ammonium was analysed according to Liddicoat et al. (1975).

Chlorophyll a was determined by the fluorescence method calibrated with the Lorenzen equation (Strickland and Parsons, 1972). The sample was homogenized for 15 s and extracted in 90% acetone in the dark and at 4°C for 15 min. In vivo fluorescence was determined on a Turner Model 10 fluorometer (Turner Associates, Palo Alto, California) after a 10 s delay.

ATP was assayed by a modification of Holm-Hansen and Booth (1966). Cells in 0.5 and 1.0 ml of culture were collected on 25 mm diameter glass fiber filters, extracted for 5 min in boiling 0.02 M, pH 7.78 tris buffer and stored frozen at -20°C until analysed, usually no more than 5 d. Extracted ATP was determined on a JRB Model 2000 ATP photometer (JBR Inc., La Jolla, California), using buffered firefly lantern extract (Sigma Chemical Co., St. Louis, Missouri) as a standard.

Cell number was determined on a Model Z_B Coulter Counter (Coulter Electronics Inc., Hialea, Florida) by taking the mean of 20 counts from two aliquots.

Experimental Procedures

Preparation of the radiosulfate stock solution. Radiosulfate, as carrier-free $H_2{}^{35}SO_4$, was obtained from New England Nuclear Corporation (Boston, Massachusetts, cat. no. NEX-042) in one ml of distilled water. To eliminate possible particulate radioactive contaminants (see also Appendix A) the solution was dialyzed (1 cm width dialysis tubing, 12,000 molecular weight cutoff, Fisher Scientific Company) against sterile, filtered distilled water for 24 h in the dark and at room temperature. The volume of distilled water was chosen such that the desired stock solution activity (100-600 μCi ml^{-1}) was achieved after equilibration. The equilibrated solution, without added sodium chloride, was sealed in 10 ml glass ampules and autoclaved for 30 min.

Immediately prior to an experiment, the ^{35}S stock solution was passed through a 12 mm diameter, 0.22 μm Millipore filter mounted in a Swinnex filter holder (Millipore Corp.). This rigorous purification procedure was superior to previous attempts to minimize the problem of retention of radioactivity by filters (Appendix A).

Sulfur-35 uptake and incorporation. The method for measuring

radiosulfate uptake is similar to that for measuring radiocarbon uptake. A population of phytoplankton was incubated for a given time with radiosulfate, filtered, washed and then the particulate rardioactivity was measured with scintillation counter.

All experiments were begun between 0900 and 1000 h. Prefiltered radiosulfate was added to 10-2000 ml of culture, depending on the experiment, to give a final radioactivity of from 1-2 μCi ml^{-1}. The same activity was not administered in each experiment because the radioactivity of the stock solution decayed with time. The sample was incubated for up to 24 h under the above light and temperature conditions. An experiment was stopped by filtering from 1.0-5.0 ml of sample, depending on the cell density in culture, diluted with 100 ml of 0.45 μm filtered seawater, through a 25 mm dramter, 1.0 μm Nuclepore filter (Nuclepore Corp., Pleasanton, California). The filtered seawater helped to reduce the radioactivity retained by the filter as did use of Nuclepore filters compared with other membrane or glass fiber filters (Appendix A). The filter was washed with 100 ml of filtered seawater, the filter chimney was removed and the filter edge was rinsed with about 20 ml of seawater from a squeeze bottle. The filter chimney and holder were rinsed in seawater prior to each filtration

to further reduce radioactive contamination of the filter (Appendix A).

Sulfur-35 uptake was determined after placing the labelled cells retained on the washed filter into glass scintillation vials containing a mixture of concentrated Spectrafluor (Amersham/Searle Corp., Oakville, Ontario), Triton X-100 (Amersham/Searle Corp.), and toluene (0.12:1.00:1.88 v/v).

Sulfur-35 incorporation was determined after treatment of the labelled cells retained on the washed filter with 10 ml of room temperature 10% (w/v) TCA for 1 min followed by 10 ml of methanol:ether (1:1, v/v) for 1 min while the filter was still mounted on the filter assembly. Chloroform is incompatible with polycarbonate Nuclepore filters and could not be used as a solvent.

A "time-zero" filter blank (Morris et al., 1971a) was determined on a sample volume equivalent to that used in the experiment, withdrawn immediately after addition of radiosulfate and treated identically to the experimental sample. The time-zero filter blank did not vary with cell concentration (Appendix A).

Rates of sulfur uptake and incorporation were calculated from:

$$U = \frac{(R_s - R_b)\ S}{R \cdot T}$$

where U = rate of sulfur uptake or incorporation (μmol S l^{-1} h^{-1}),

R_s = the radioactivity of the filtered sample (d.p.m. ml^{-1}),

R_b = the radioactivity of the "time-zero" filter blank (d.p.m. ml^{-1}),

S = the sulfate sulfur concentration of seawater (7.27×10^4 μmol S l^{-1}),

R = the radioactivity of the incubation medium (d.p.m. ml^{-1}), and

T = the incubation time (h) during the period of light.

A factor was not used to correct for isotopic discrimination of ^{32}S vs. ^{35}S. However, the degree of discrimination should not be greater than the maximum of 4% found for ^{32}S vs. ^{34}S (Smejkal et al., 1971; Jordan and Peterson, 1978).

The ionic sulfate concentration of the culture medium was calculated from a sulfate:chloride ratio of 0.1400 (Culkin, 1965), using a salinity of 30‰.

Carbon-14 uptake. The radioactivity of ^{14}C and ^{35}S was measured in separate aliquots as dual labelling was not feasible due to the similar energy of both radioisotopes. Photosynthetic ^{14}C fixation was determined by collecting cells from a 2.0 ml aliquot culture medium incubated with 0.055 μCi ml^{-1} of 0.22 μm prefiltered radiocarbonate (Atomic Energy of Canada, Ltd., Ottawa, Ontario) on a

25 mm diameter, 1.0 µm Nuclepore filter. The filter was washed and placed in scintillation fluor as described for the ^{35}S experiments. Rates of carbon uptake (µmol C l^{-1} h^{-1}) were calculated according to Strickland and Parsons (1972), using 2.04 x 10^3 µmol C l^{-1} for the concentration of total carbon in seawater.

Scintillation spectrometry. Radioactivity was measured on a Packard Tri Carb Model 3380 (Academy Instruments, Inc., Scarborough, Ontario) or a Searle Mark II Model 6847 (Searle Instrumentation, Oakville, Ontario) scintillation spectrometer. Samples were counted until at least 5000 counts were obtained, resulting in a standard deviation of ± 1.5%. Counts were corrected for quenching and for decay of the ^{35}S isotope. Counting efficiency was determined by the channels ratio method using an external standard and ^{14}C quench standards (Searle Instrumentation).

Hydrolysis of the protein fraction. *Thalassiosira weissflogii* from the exponential and stationary phases was labelled with 2.0 µCi ml^{-1} of radiosulfate for 24 h and 5 d, respectively. Cells were collected by centrifugation for 5 min at 4,000 x *g*, and extracted while in the centrifuge tube with 5.0 ml of room temperature 10% (w/w) TCA for 10 min, and 5.0 ml of ether:methanol (1:1)

for 10 min. The residue was boiled for 30 min in 50 ml of distilled water, then homogenized for 3 min and extracted for 60 min in 5.0 ml of chloroform:methanol (2:1). Solutions were evaporated to dryness at 70°C in scintillation vials under a stream of air, and reconstituted with fluor prior to scintillation counting.

Hydrolysis of the fraction obtained above, containing mainly protein, was carried out in 2 ml of constant boiling 6 N HCl under a nitrogen atmosphere in sealed ampules at 110°C for 24 h (Moore and Stein, 1951). The hydrolysate was evaporated at 70°C under a stream of air to drive off the HCl, and was then reconstituted with distilled water. Chromatography was achieved on a 5 x 50 mm column of AG 50W-x8 (100-200 mesh) cation exchange resin in the H^+ form (Bio-Rad Labs, Dickman, California). The column was washed with 5 bed volumes of distilled water. Five bed volumes of 2 N NH_4OH were then added to elute amino acids. Solutions were evaporated to dryness as above prior to scintillation counting.

Sephadex gel chromatography of the protein fraction. Thalassiosira weissflogii from the exponential phase was labelled with 0.50 $\mu Ci\ ml^{-1}$ radiosulfate for 24 h. Cells were collected by centrifugation for 5 min at 4,000 x g, using a Sorvall Model RC2-B centrifuge (Ivan Sorvall Inc.,

Newton, Connecticut). The pellet was washed 4 times with filtered seawater, extracted 3 times for 10 min each at room temperature with 5.0 ml of 10% (w/v) TCA, and 3 times for 10 min each at room temperature with 5.0 ml of methanol:ether (1:1). The pellet was sonicated at 30 KHz (Sonipak processor, Model Sp-201-30, Prosonics, Inc., Grayslake, Illinois) for 10 min in an ice bath, then extracted for 12 h in 3.0 ml of 0.2 N NaOH. The supernate containing the extracted protein was adjusted to pH 9.5 with glacial acetic acid, and made to 7.0 ml with pH 9.5 borate-sodium hydroxide buffer (Gomori, 1955). A 3.5 ml aliquot was then fractionated on a 2.5 cm x 33 cm column of Sephadex G-25 coarse gel (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been equilibrated with the same buffer. The flow rate was 0.5 ml min^{-1} and 2.0 ml fractions were collected. Blue dextran 2000 (Pharmacia Fine Chemicals) was used to determine the void volume.

Gentamycin treatment. The antibiotic, gentamycin (Caskey, 1973) was added to a mixed culture of marine bacteria started by incubating 25 ml of 0.45 µm filtered seawater plus 100 ml of sterile f/2 medium containing glucose (1%), tryptone (1%) and yeast extract (0.5%), in the dark at 20°C. A dense bacterial culture was obtained in 2 days. Bacterial growth was measured optically with

a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., New York) fitted with a blue filter. The concentrations of gentamycin (Sigma Chemical Co., St. Louis, Missouri) used were: 0, 20, 50, 60, 100 and 200 μg ml^{-1}. Gentamycin and radiosulfate were added simultaneously at the start of an experiment.

Cycloheximide treatment: Cycloheximide (Sigma Chemical Co.), an inhibitor of protein synthesis in eucaryotes (Pestka, 1977), was added to a steady-state culture of T. weissflogii grown in a nitrate-limited chemostat to give a final concentration of 25 μg ml^{-1}.

Field Procedures

North West Arm. Samples were collected at 0930 h with a 5 l Niskin bottle at 2 m from the Halifax Police Association Yacht Club pier, North West Arm. Ambient water temperature was 13°C. Within 15 min after collection, the water was prefiltered through a 150 μm mesh and placed into 150 ml incubation bottles containing 1.30 μCi ml^{-1} of radiosulfate. After incubation for 24 h at 10°C under a bank of cool white fluorescent bulbs with about 40 μE m^{-2} s^{-1} of constant illumination, 150 ml of sample were filtered for determination of ^{35}S incorporation. Carbon-14 uptake was measured under the same conditions with 0.03 μCi ml^{-1} of radiocarbonate.

Peru. Experiments were carried out during Legs 1 and 2 (October 28 to November 12, 1977) of the Project ICANE (Investigacion Cooperative de la Anchoveta y su Ecosistoma), funded in part by the Canadian International Development Agency (CIDA).

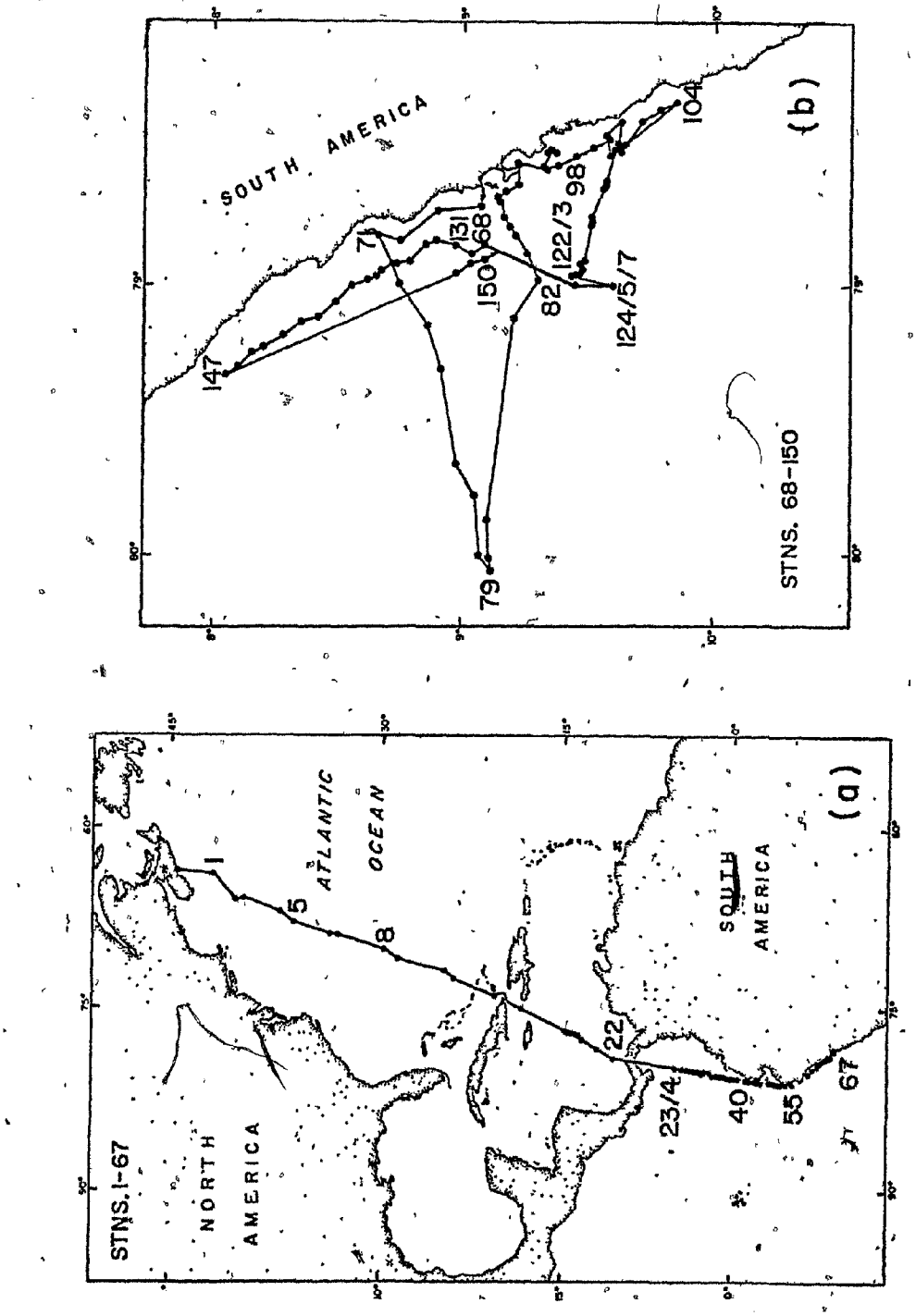
Samples were collected from the depths corresponding to the 100, 50, 25, 10 and 1% light levels at stations 23, 40, 48, 56, 71 and 75 (Fig. 1). Additional stations where ^{14}C incorporation was measured in the presence and absence of gentamycin, are reported in a data report (Bedford Institute of Oceanography Data Report, in preparation). Samples were screened through a 202 µm mesh at all stations except 71 and 75. They were then incubated for 24 h in 150 ml bottles containing 2.6 µCi ml^{-1} of radiosulfate in simulated in situ deck incubators cooled by surface water. Sulfur-35 incorporation was determined on 150 ml of sample as described above.

Specific growth rates were calculated from ^{14}C uptake data and C:chl a weight ratios (Eppley, 1972) using the equation:

$$\mu = 3.32 \log \left(\frac{C\ chl^{-1} + mg\ C\ mg\ chl^{-1}\ d^{-1}}{C\ chl^{-1}} \right)$$

C:chl a ratios were derived from linear regressions

Figure 1. Cruise track of the CSS Baffin.
(a) Stations 1-67 and (b) stations 68-150.


SOUTH AMERICA
(b)
147
71
131
68
150
79
82
122/3
98
124/5/7
104
STNS. 68-150
STNS. 1-67
NORTH AMERICA
ATLANTIC OCEAN
SOUTH AMERICA
(a)
1
5
8
22
23/4
40
55
67

of POC on chlorophyll a of the same water samples (Banse, 1977). Two regressions were found corresponding to Stations 23, 48, 71 and 75 (1), and Station 56 (2):

$$POC = 102\ chl + 103 \qquad (r = 0.96,\ n = 20) \qquad (1)$$

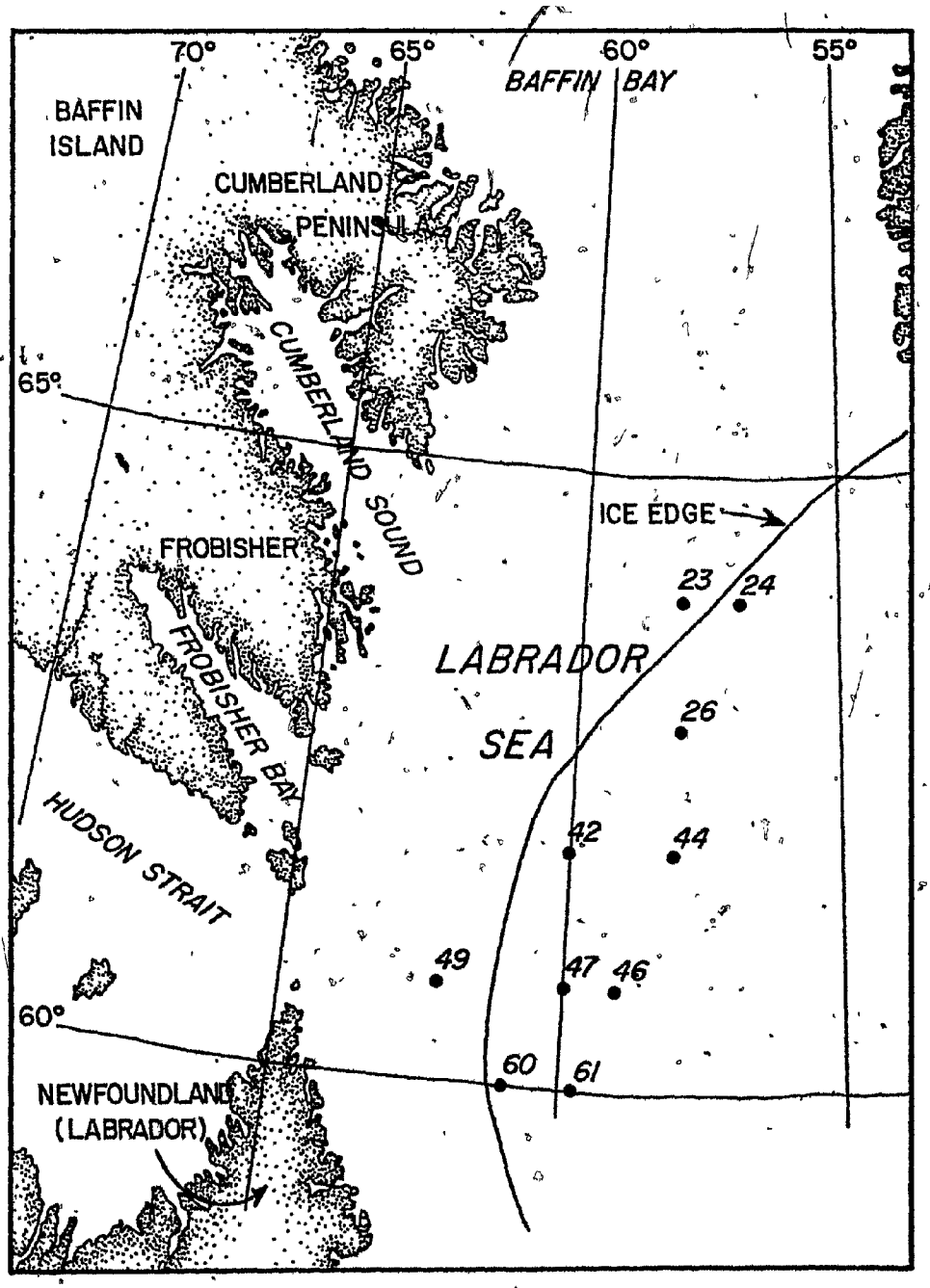
$$POC = 30\ chl + 96 \qquad (r = 0.99,\ n = 4) \qquad (2)$$

where POC = mg C m^{-3} and chl = mg Chl a m^{-3}.

Rates of photosynthetic carbon fixation, chlorophyll a and POC were obtained from Doe (1978).

Davis Strait. Sulfur-35 uptake experiments were carried out in the Davis Strait by Mark Huntley in conjunction with grazing experiments during Cruise 78-1, funded by ESSO Resources, Ltd., Aquitaine Co. of Canada, Ltd., and Canada Cities Services Ltd. (MacLaren Marex, 1979a; 1979b). Samples were collected from 10 m at MacLaren station numbers 46 and 42 in April, 1978, and at stations 24 and 23 in May, 1978 (Fig. 2). After screening through a 153 µm mesh, 1.9 l of sample was inoculated with radiosulfate to give an activity of about 0.3 µCi ml^{-1}, and incubated for 24-66 h at 10°C under about 200 µE m^{-2} s^{-1} of constant illumination provided by a bank of cool white fluorescent lamps. Ambient temperature was about 0°C. Sulfate uptake was determined by filtering 250 ml of sample as described above.

Figure 2. MacLaren Marex sampling area and station numbers in the Davis Strait.


70°
65°
60°
55°
BAFFIN BAY
BAFFIN ISLAND
CUMBERLAND PENINSULA
CUMBERLAND SOUND
65°
ICE EDGE
FROBISHER
FROBISHER BAY
23
24
LABRADOR SEA
26
HUDSON STRAIT
42
44
49
47
46
60°
60
61
NEWFOUNDLAND (LABRADOR)

RESULTS

Measurement of ^{35}S incorporation

Sulfur-35 labelled cells are treated while on the filter with TCA to precipitate protein within the cell and to solubilize and remove low molecular weight sulfur-containing compounds from the cell. Treatment of ^{35}S-labelled Thalassiosira weissflogii with 10 ml of 10% TCA at the boiling point removed about 10% more activity than did TCA at room temperature (Table 1). Nevertheless, because it is more practical at sea, TCA was used at room temperature throughout this study. Protein is precipitated within the first 3 seconds, as virtually the same activity was observed after the TCA remained in contact with the labelled cells for up to 7 min (Table 1). Extraction with TCA followed by methanol:ether removed more radioactivity than did TCA alone.

Repeated extractions of the cells with TCA and methanol:ether for one minute each while on the filter did not remove more than about 50% of the total ^{35}S taken up (Table 2). However, when repeated extractions for 10 min each were carried out in a centrifuge tube, about 60% of the radioactivity was removed, leaving about 40% in the protein fraction (Table 3). Protein is not lost from the cell after treatment with TCA and methanol:ether while on the filter. Colorimetric analysis showed that the same

Table 1. Effect of extracting ^{35}S-labelled T. weissflogii with 10% TCA at 20°C or at the boiling point for varying times upon the radioactivity remaining in the TCA insoluble fraction expressed as a % ± S.D. of the total radiosulfate taken up.

Extraction time (min)	% Radioactivity in TCA insoluble fraction	
	TCA at 20°C	TCA at boiling point
0.05	74.9 ± 0.9	63.4 ± 1.3
0.50	74.9 ± 1.2	62.9 ± 0.8
1.00	73.5 ± 2.7	67.0 ± 1.6
2.00	73.7 ± 1.3	64.0 ± 1.4
3.00	74.8 ± 1.4	63.3 ± 0.9
5.00	74.5 ± 1.8	57.1 ± 2.0
7.00	74.6 ± 1.0	64.4 ± 1.3

Table 2. Factorial extraction of ^{35}S-labelled T. weissflagii with increasing volumes of methanol:ether (1:1, v/v) and 10% TCA at 20°C for 1 min each. The values shown are the percent (± S.D.) of the total radioactivity taken up that remained in the TCA plus methanol:ether insoluble fraction.

Volumes of TCA	Volumes of methanol:ether			
	10 ml x 1	10 ml x 2	10 ml x 3	10 ml x 4
10 ml x 1	52.4 ± 1.4	51.2 ± 2.2	50.6 ± 0.9	51.3 ± 1.2
10 ml x 2	53.4 ± 0.8	50.1 ± 1.4	48.9 ± 1.5	49.1 ± 1.4
10 ml x 3	51.4 ± 2.7	50.0 ± 1.2	49.2 ± 1.2	48.5 ± 1.4
10 ml x 4	49.3 ± 0.8	46.8 ± 1.8	48.4 ± 1.2	46.2 ± 1.5

Table 3. Sulfur-35 (d.p.m.) and percent of the total radioactivity remaining in fractions obtained by extraction of ^{35}S-labelled T. weissflogii which were ruptured by sonication. The sodium hydroxide soluble fraction was subsequently chromatographed on a Sephadex G-25 column (see Fig. 3).

Fraction	Extraction number	d.p.m.	Percent of total
TCA soluble	1	174251	
	2	25602	
	3	6916	
	Total	206769	55.4
Methanol:ether soluble	1	10714	
	2	2314	
	3	546	
	Total	13574	3.6
Sodium hydroxide soluble	-	137127	36.7
Pellet residue	-	16094	4.3
Total	-	373564	100.0

quantity of protein was recovered from the filter as from centrifugation of the same number of cells.

Association of ^{35}S with the protein fraction

Three approaches were taken to determine if the incorporated ^{35}S is associated with the protein fraction: 1) inhibition of protein synthesis with cycloheximide, 2) measurement of ^{35}S associated with amino acids derived from protein hydrolysis, and 3) Sephadex gel chromatography of the ^{35}S-labelled fraction.

Cycloheximide inhibition. Cycloheximide is a known inhibitor of protein synthesis in eucaryotes (Pestka, 1977). I added the inhibitor to cultures of T. weissflogii pre-conditioned in a nitrate-limited chemostat and I then measured protein concentration and ^{35}S incorporation under conditions that would favour (883 µM nitrate added) or disfavour (nitrate absent) protein synthesis. Cycloheximide inhibited protein synthesis, slowed ^{35}S uptake, and virtually stopped ^{35}S incorporation (Table 4).

Hydrolysis of the protein fraction. The distribution of ^{35}S-labelled compounds in T. weissflogii is shown in Table 5. Results from exponential and stationary phase cells are not significantly different. The chloroform: methanol soluble (sulfolipids remaining after extraction with methanol:ether) and boiling water soluble (sulfated

Table 4. Effect of adding cycloheximide (25 μg ml^{-1}) and nitrate (883 μM) on cellular protein, sulfur uptake, sulfur incorporation, and photosynthetic carbon uptake by T. weissflogii preconditioned in a nitrate-limited chemostat. Each value, shown with ± S.D., is the mean of three experiments. The parameters for each experiment were analyzed in triplicate.

	(-) Cycloheximide		(+) Cycloheximide	
Parameter	(-) Nitrate	(+) Nitrate	(-) Nitrate	(+) Nitrate
Initial protein (pg cell^{-1})	104.0 ± 7.0	104.0 ± 7.0	104.0 ± 7.0	104.0 ± 7.0
Final protein (pg cell^{-1})	90.0 ± 11.0	120.0 ± 10.0	109.0 ± 6.0	110.0 ± 9.0
S uptake (fmol cell^{-1} h^{-1})	6.86 ± 3.31	12.66 ± 2.31	1.43 ± 0.25	2.68 ± 0.12
S incorporation (fmol cell^{-1} h^{-1})	3.18 ± 1.19	6.55 ± 0.69	0.25 ± 0.06	0.31 ± 0.06
C uptake (fmol cell^{-1} h^{-1})	331.0 ± 65.0	211.0 ± 7.0	388.0 ± 50.0	393.0 ± 42.0

Table 5. Percent (± S.D.) of total ^{35}S uptake in fractions obtained by extraction of ^{35}S-labelled T. weissflogii cells harvested during the exponential or stationary phases of growth, and ruptured by homogenization. The protein fraction was then chromatographed on an AG 50W-x8 column to yield the distilled water and ammonium hydroxide eluates, plus the miscellaneous fractions remaining in the pellet, vial, and extruded resin.

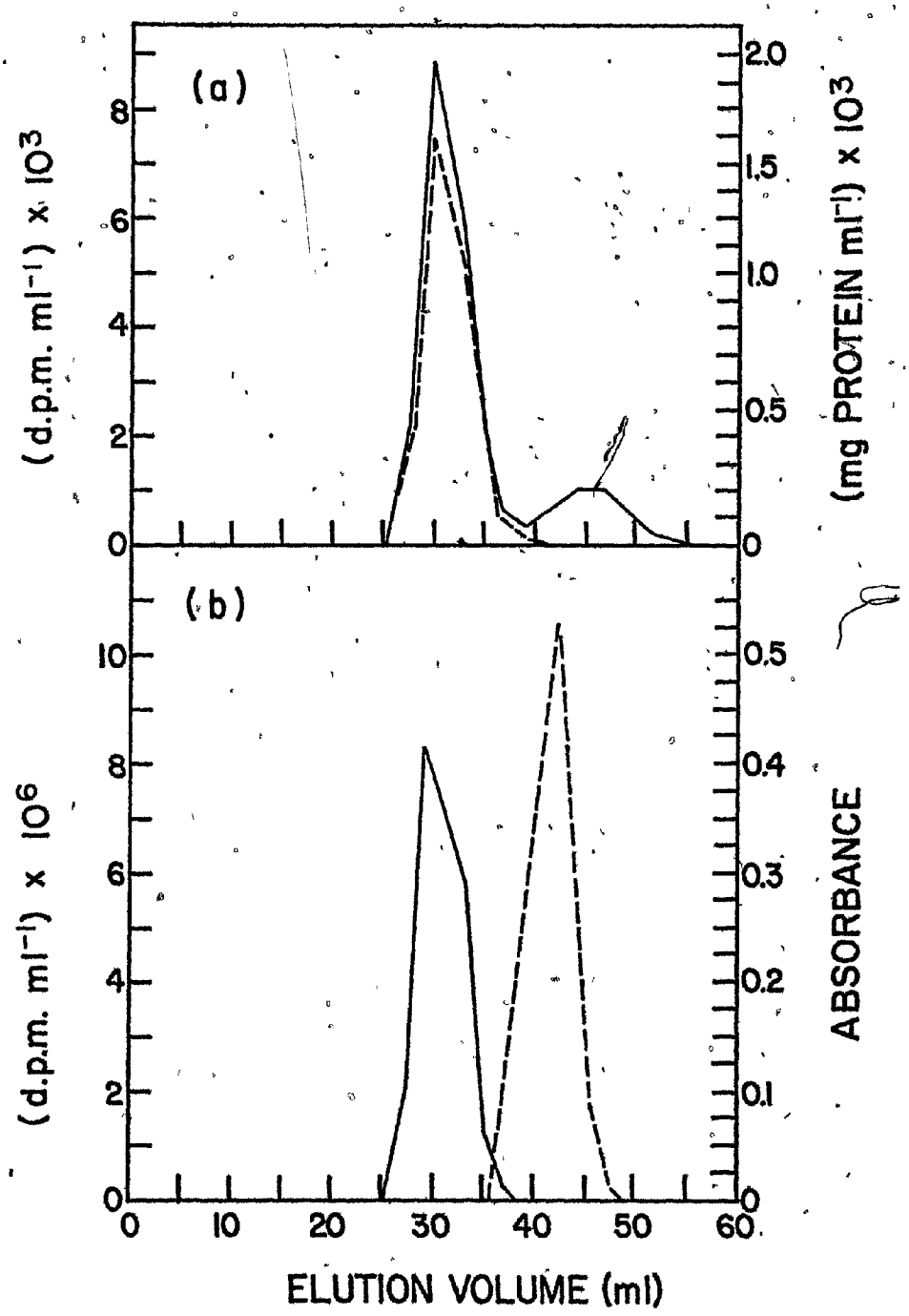
Fraction	Percent radioactivity	
	Exponential phase	Stationary phase
Total uptake	100.0 ± 0.0	100.0 ± 0.0
TCA + methanol:ether soluble	44.5 ± 5.2	45.4 ± 1.2
Boiling water soluble	4.5 ± 2.8	6.4 ± 2.9
Chloroform:methanol soluble	1.5 ± 1.6	1.3 ± 1.2
Protein residue	(47.8 ± 1.7)	(50.8 ± 13.6)
Distilled water eluate	22.7 ± 18.0	20.1 ± 5.9
Ammonium hydroxide eluate	12.3 ± 3.4	9.9 ± 1.0
Extruded resin	0.4 ± 0.2	0.3 ± 0.1
Residue remaining in pellet	0.3 ± 0.1	0.2 ± 0.1
Activity remaining in vial	0.6 ± 0.5	0.1 ± 0.1
Total Recovery	86.8	83.7

polysaccharide) compounds comprise a low percentage of the total ^{35}S taken up.

The TCA plus methanol:ether insoluble (protein) fraction of T. weissflogii accounted for about 50% of the total activity taken up (Table 5). However, when this fraction was hydrolyzed and fractionated on an AG 50-x8 column, only about 11% of the total ^{35}S in the cells, or about 25% of the radioactivity in the hydrolysate was recovered in the ammonium hydroxide (amino acid) fraction. Almost twice as much radioactivity was found in the uncharacterized distilled water eluate (Table 5). About 2.4% of the nitrogen added to the chromatography column also appeared in the distilled water eluate.

Sephadex gel chromatography of the protein fraction. The protein fraction of ^{35}S-labelled T. weissflogii was solubilized and fractionated on a Sephadex G-25 column. Peaks of radioactivity and protein concentration coincided and were eluted at the exclusion volume of the column (Fig. 3a). A secondary peak of ^{35}S was eluted at about 46 ml (Fig. 3a), while the radiosulfate salt peak occurred at about 41 ml (Fig. 3b). Recoveries of 98% and 95% were achieved for protein and radioactivity, respectively.

Figure 3. Column chromatography of (a) ^{35}S compounds (solid line) and protein (dashed line) in the TCA plus methanol:ether insoluble fraction of T. weissflogii, obtained by sequential extraction of ^{35}S-labelled cells, and (b) blue dextran 2000 (solid line) and radiosulfate (dashed line). A 2.5 x 33 cm column of Sephadex G-25 coarse gel was eluted with pH 9.5 borate-sodium hydroxide buffer at 0.5 ml min^{-1}.


(a)
(b)
(d.p.m. ml⁻¹) x 10³
(d.p.m. ml⁻¹) x 10⁶
(mg PROTEIN ml⁻¹) x 10³
ABSORBANCE
ELUTION VOLUME (ml)
0
2
4
6
8
10
0.1
0.2
0.3
0.4
0.5
1.0
1.5
2.0
10
20
30
40
50
60

Comparison between rates of ^{35}S incorporation and changes in biomass

Time-course of ^{35}S incorporation over 24 h. Over a 24 h cycle ^{35}S incorporation by T. weissflogii paralleled cell number with a linear increase during the light period and a slight increase in the dark (Fig. 4a). Sulfur-35 incorporation by a natural population of unknown composition from Gatun Lake, Panama, exhibited a similar diel cycle (not shown). Uptake of ^{35}S and labelling of sulfur compounds other than protein followed a similar pattern over a 24 h cycle (Fig. 4b), although linearity in the light was not evident in that experiment. The activity of the methanol: ether soluble compounds peaked at about 7 h of incubation then steadily declined.

Time-course of ^{35}S incorporation over 7 days. Daily measurements of ^{35}S incorporation and biomass were made over the growth cycle in batch culture with T. weissflogii growing in the continuous presence of radiosulfate and with nitrate at f/1 (= 1766 µM), f/2 (= 883 µM), f/10 (= 177 µM) and f/100 (= 18 µM). Biomass parameters for growth with nitrate at f/2 and f/100 are compared in Fig. 5. Growth curves for f/1 and f/10 were similar to those for f/2. The yield of cells was less at f/100 than at the other nitrate concentrations as indicated by all biomass indices. Significant positive correlations were found

Figure 4. Time-course over 24 h of (a) ^{35}S incorporation (●), cell concentration (■), and (b) ^{35}S uptake (■), TCA soluble (▲), methanol:ether soluble (◆), and TCA-methanol:ether insoluble (●) ^{35}S-labelled fractions of T. weissflogii. (a) and (b) represent separate experiments. The dark period is indicated by a solid bar on the x axis.

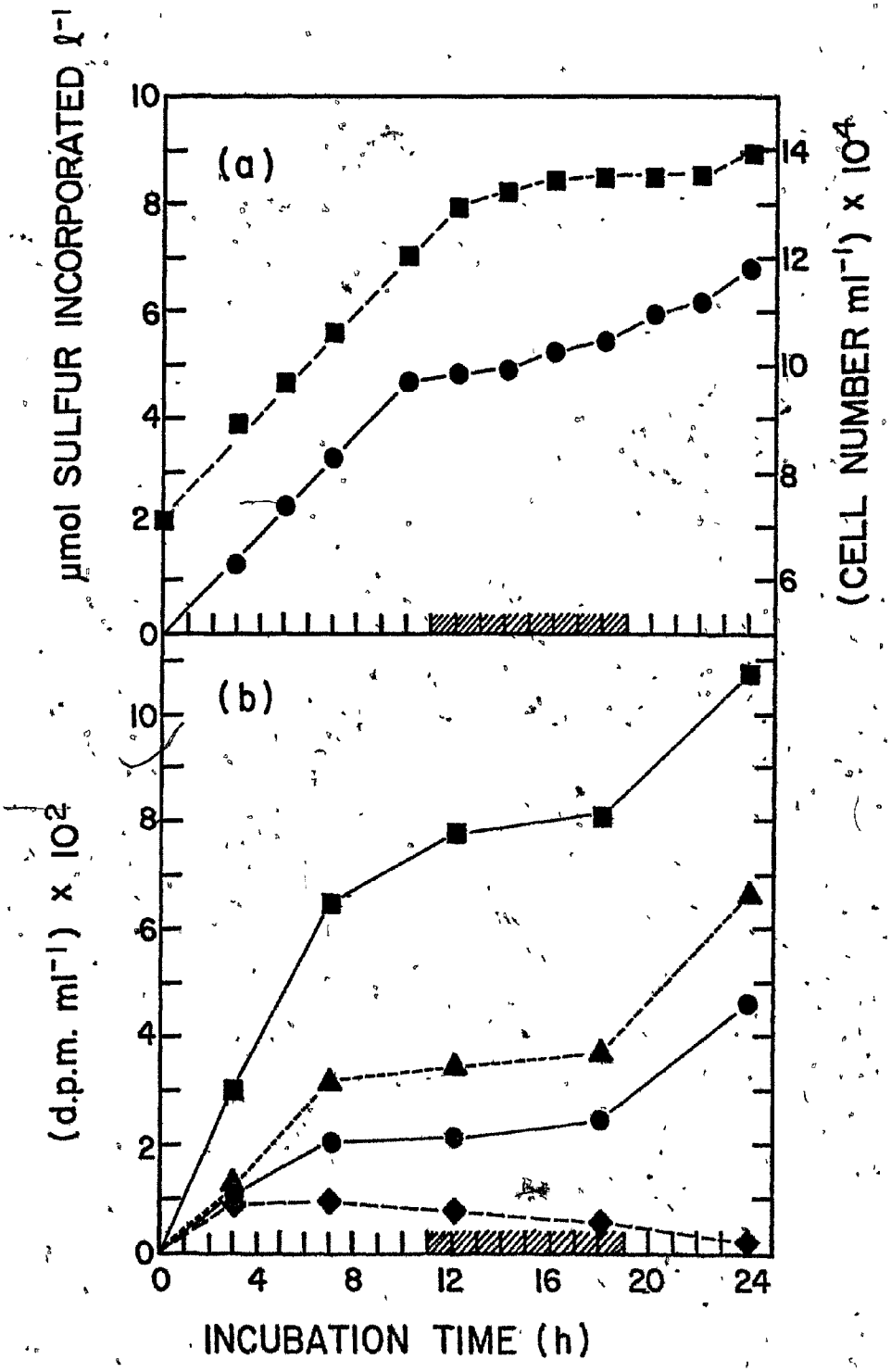
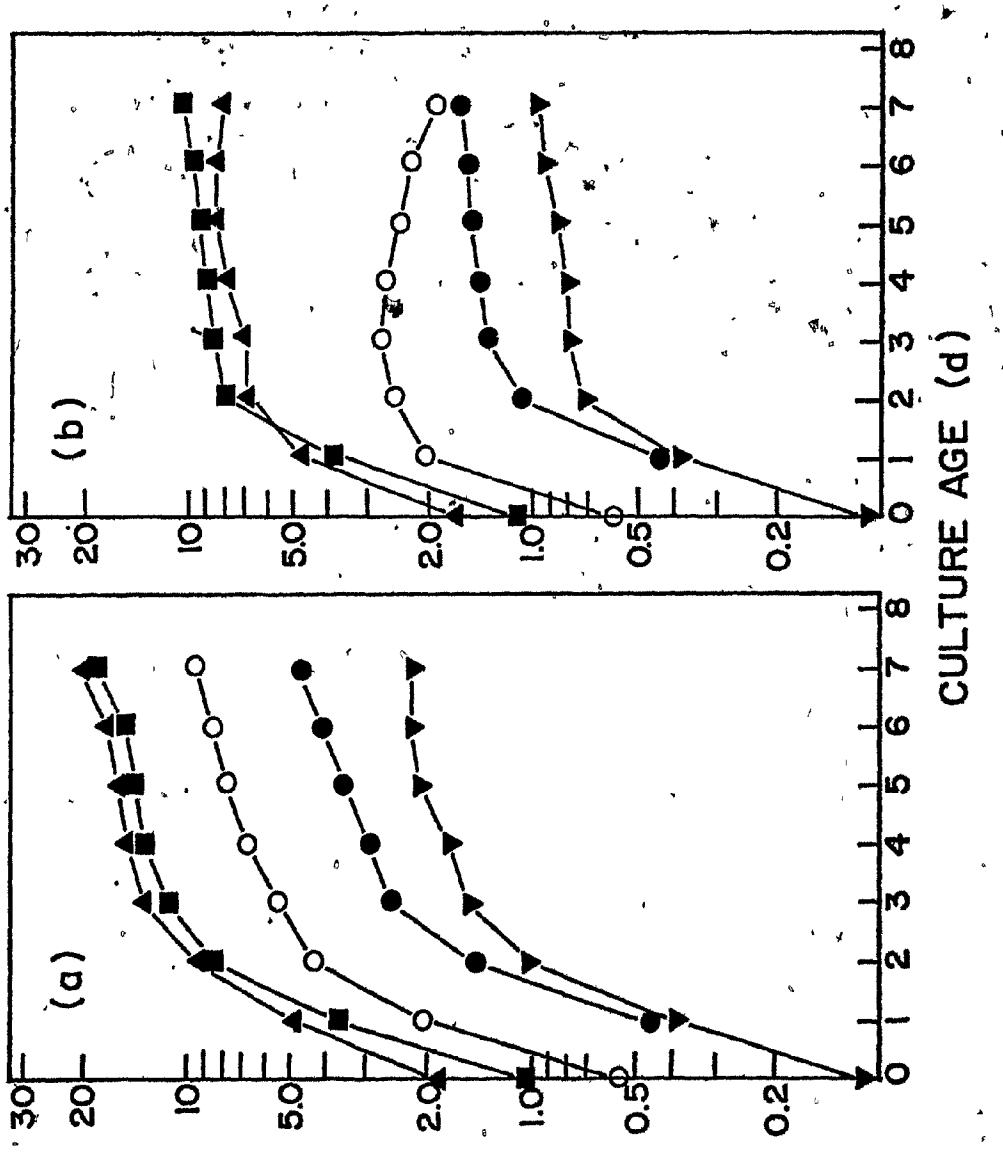

µmol SULFUR INCORPORATED l⁻¹
(CELL NUMBER ml⁻¹) x 10⁴
(a)
(b)
(d.p.m. ml⁻¹) x 10²
INCUBATION TIME (h)
0
2
4
6
8
10
12
14
16
20
24

Figure 5. Time-course over 7 d of sulfur incorporation (●) as (nmol S ml^{-1}) x 10, cell concentration (■) as (cells ml^{-1}) X 10^4, protein (▲) as µg protein ml^{-1}, chlorophyll *a* (○) as (ng chl *a* ml^{-1}) X 10, and relative *in vivo* fluorescence units (▼) by *T. weissflogii* growing in batch culture with nitrate supplied at (a) 883 µM and (b) 18 µM.


(a)
(b)
30
20
10
5.0
2.0
1.0
0.5
0.2
0 1 2 3 4 5 6 7 8
CULTURE AGE (d)

between ^{35}S incorporation and cell number, protein, chlorophyll a and in vivo fluoresence over the growth cycle at the four nitrate concentrations (Fig. 6). The highest correlations were between ^{35}S incorporation and protein concentration at the individual nitrate concentrations. But when points from all nitrate concentrations were combined, the highest correlation was between ^{35}S incorporation and in vivo fluorescence (Fig. 6).

Comparison between rates of ^{35}S incorporation and ^{14}C uptake

Effect of nitrate concentration. When ^{35}S incorporation is measured in the field, populations of diverse physiological state are incubated for 24 h periods. This situation was simulated in the laboratory with T. weissflogii growing in batch culture at four nitrate concentrations (Fig.7).

When expressed on a per litre basis, the rate of ^{35}S incorporation reached a maximum on day 4 for growth in nitrate at f/2, on day 3 for f/25 and f/50, and on day 2 for f/100 (Fig. 8). The peaks of sulfur incorporation occurred at the transition between the exponential and stationary growth phases (Fig. 7). Rates of ^{14}C uptake, on the other hand, were maximum 24 h later than ^{35}S incorporation (Fig. 8b,c,d) except for cells grown in f/2 when both maxima occurred on the same day (Fig. 8a).

When expressed on a per cell basis, rates of ^{35}S

Figure 6. Regressions of ^{35}S incorporation of T. weissflogii on (a) chlorophyll a, y = 14.2x + 61.2, r = 0.923, (b) in vivo fluorescence, y = 6.0x + 0.9, r = 0.975, (c) cell concentration, y = 83.8x - 131.7, r = 0.941, and (d) protein, y = 78.3x - 115.2, r = 0.961. Nitrate was supplied to the batch culture at 1766 µM (●), 883 µM (■), 177 µM (○), and 18 µM (▲).

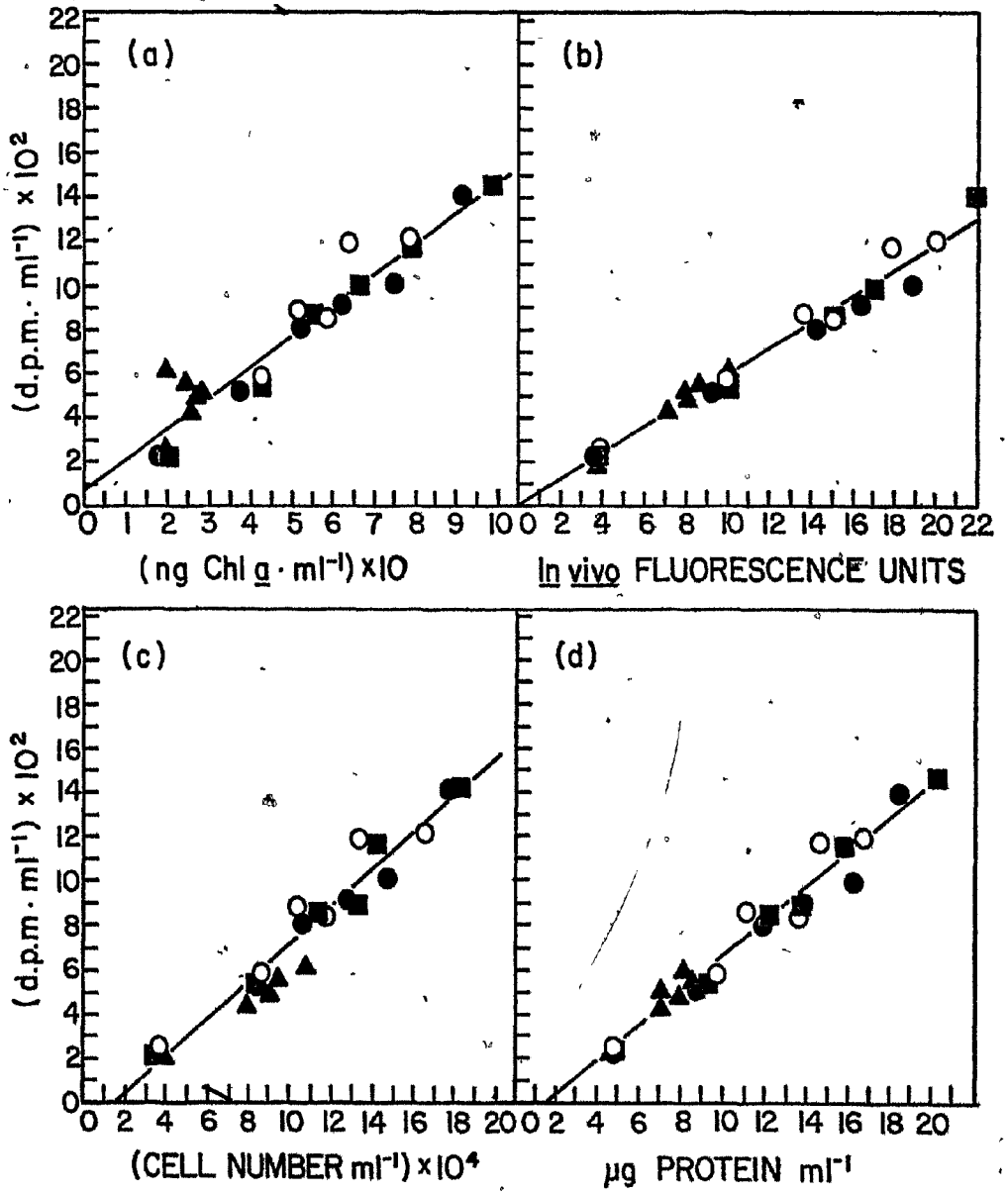
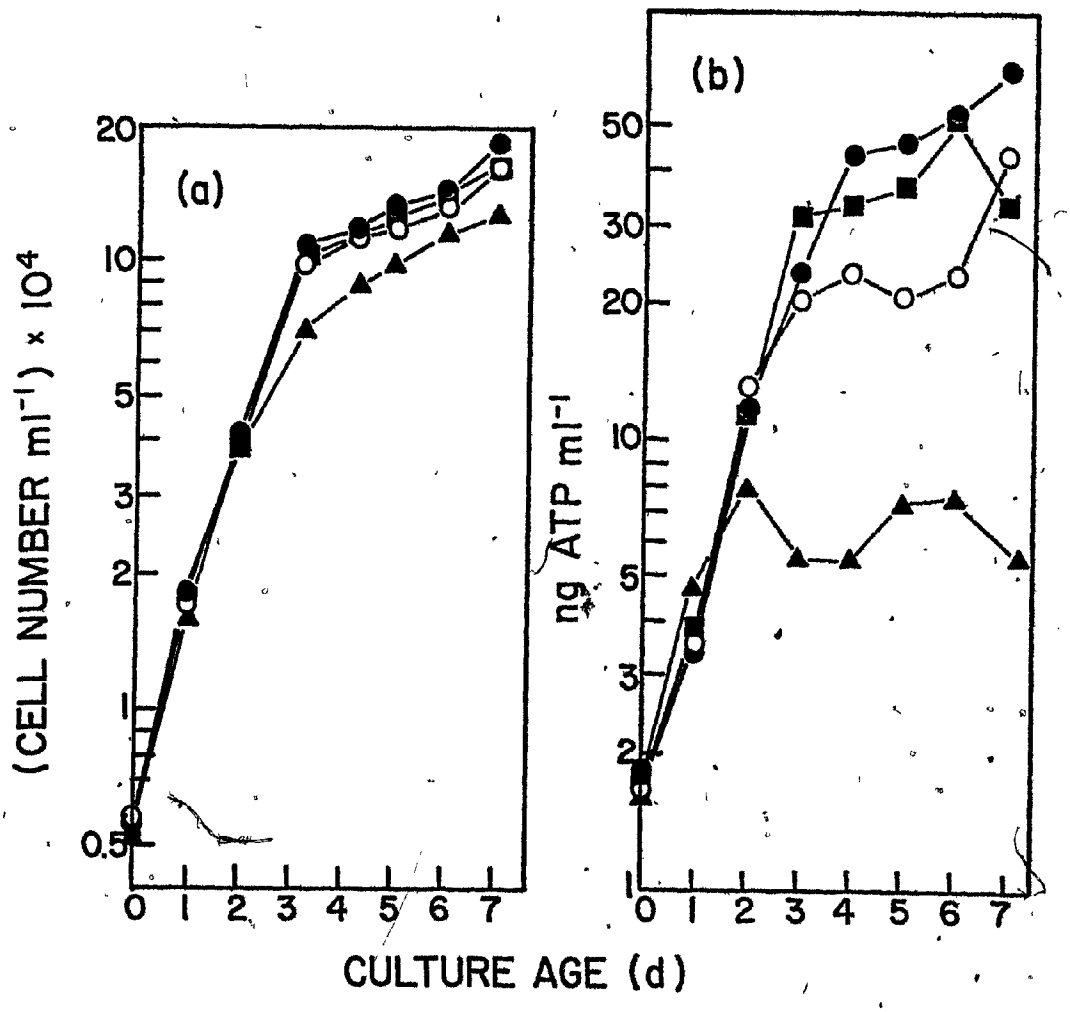

(a)
(b)
(c)
(d)
(d.p.m. · ml⁻¹) x 10²
(ng Chl a · ml⁻¹) x10
In vivo FLUORESCENCE UNITS
(CELL NUMBER ml⁻¹) x10⁴
µg PROTEIN ml⁻¹

Figure 7 (in two parts). Growth of T. weissflogii in batch culture with nitrate supplied at 883 µM (●), 70 µM (■), 35 µM (○), and 18 µM (▲). (a) Cell concentration, (b) ATP, (c) chlorophyll a, and (d) protein.


(a)
(b)
(CELL NUMBER ml⁻¹) x 10⁴
ng ATP ml⁻¹
CULTURE AGE (d)
0.5
1
2
3
5
10
20
30
50
0 1 2 3 4 5 6 7

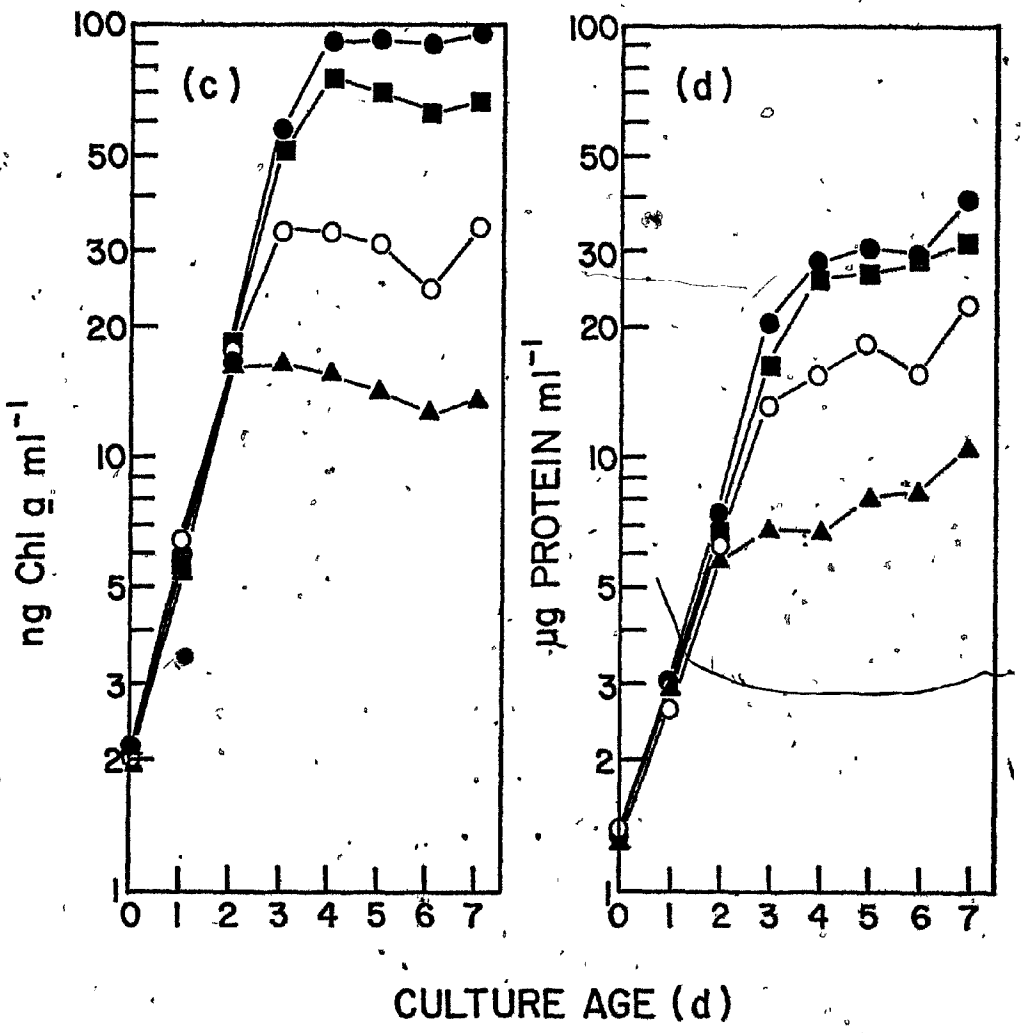
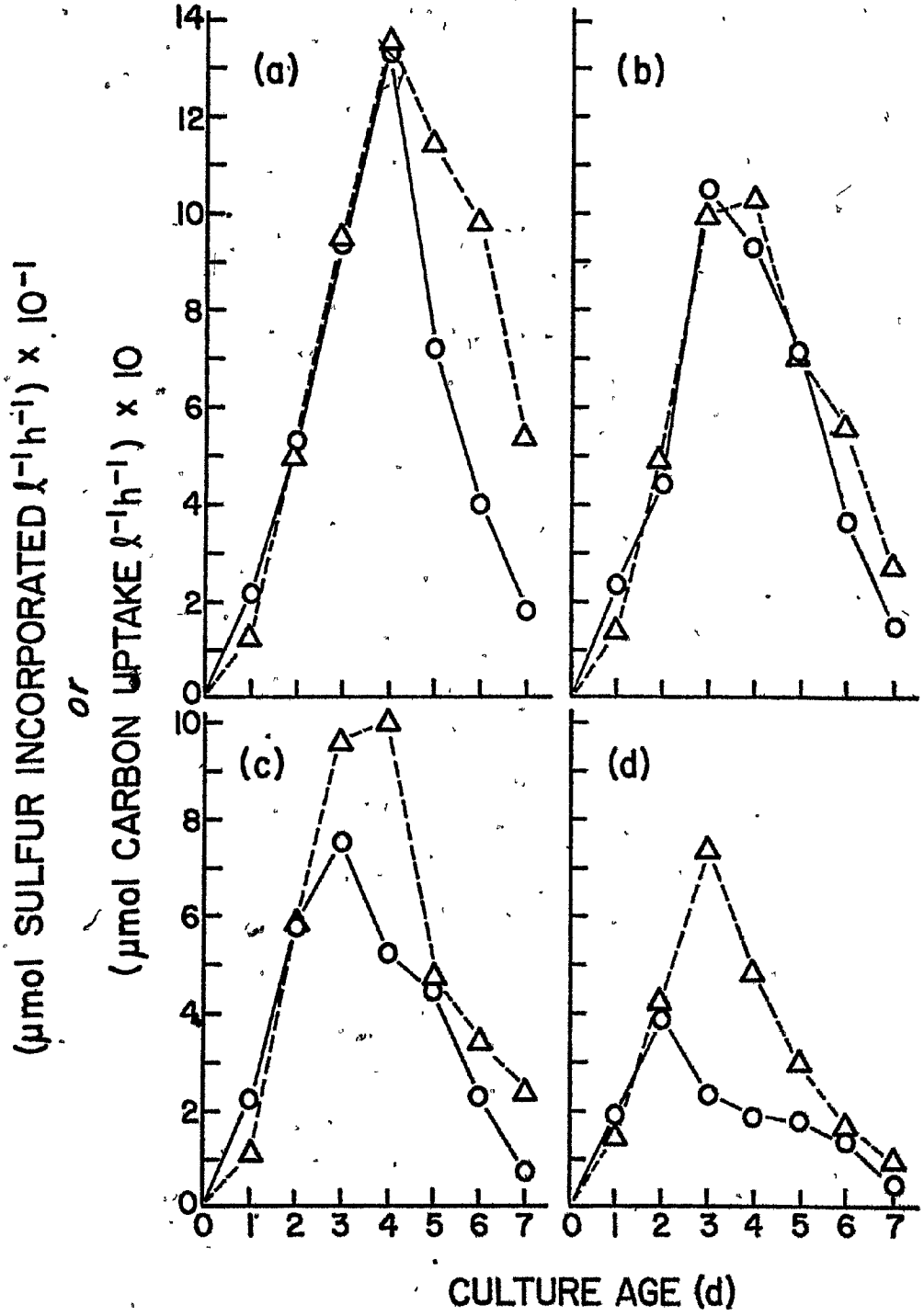

(c)
(d)
ng Chl a ml⁻¹
µg PROTEIN ml⁻¹
100
50
30
20
10
5
3
2
1
0 1 2 3 4 5 6 7
CULTURE AGE (d)

Figure 8. Rates of sulfur incorporation (○) and photosynthetic carbon uptake (△) expressed on per litre basis by T. weissflogii growing in batch culture with nitrate supplied at (a) 883 µM, (b) 70 µM, (c) 35 µM, and (d) 18 µM.


(a)
(b)
(c)
(d)
(µmol SULFUR INCORPORATED ℓ⁻¹h⁻¹) x 10⁻¹
or
(µmol CARBON UPTAKE ℓ⁻¹h⁻¹) x 10
CULTURE AGE (d)

incorporation and ^{14}C uptake generally declined over the growth cycle (Fig. 9). The initial increase in rates of carbon uptake from day 1 to day 2 was generally not seen in later experiments.

Comparison among four species. Representative species (T. weissflogii, Amphidinium carteri, Dunaliella tertiolecta and Pavlova lutheri) of the four major classes of marine phytoplankton were grown in batch culture with nitrate supplied at f/100 (Fig. 10). Nitrogen deficiency is suggested for each species during the stationary phase by the rapid decline in chlorophyll a contentration.

The pattern of ^{35}S incorporation per unit protein (Fig. 11) or per cell is similar in the four species studied, with decreasing rates from exponential growth to the stationary phase. Interspecific rates were of the same order of magnitude, but showed no obvious trend with cell size. Rates of ^{35}S incorporation per cell generally reflected the changes in protein per cell during growth in batch culture for T. weissflogii, D. tertiolecta and P. lutheri (Fig. 12a,c,d), while A. carteri (Fig. 12b) showed an anomalous pattern for protein per cell during the late stationary phase.

Effect of incubation in darkness on ^{35}S incorporation

Batch culture. Daily aliquots of T. weissflogii

Figure 9. Rates of sulfur incorporation (○) and photosynthetic carbon uptake (△) expressed on a per cell basis by T. weissflogii growing in batch culture with nitrate supplied at: (a) 883 µM, (b) 70 µM, (c) 35 µM, and (d) 18 µM.

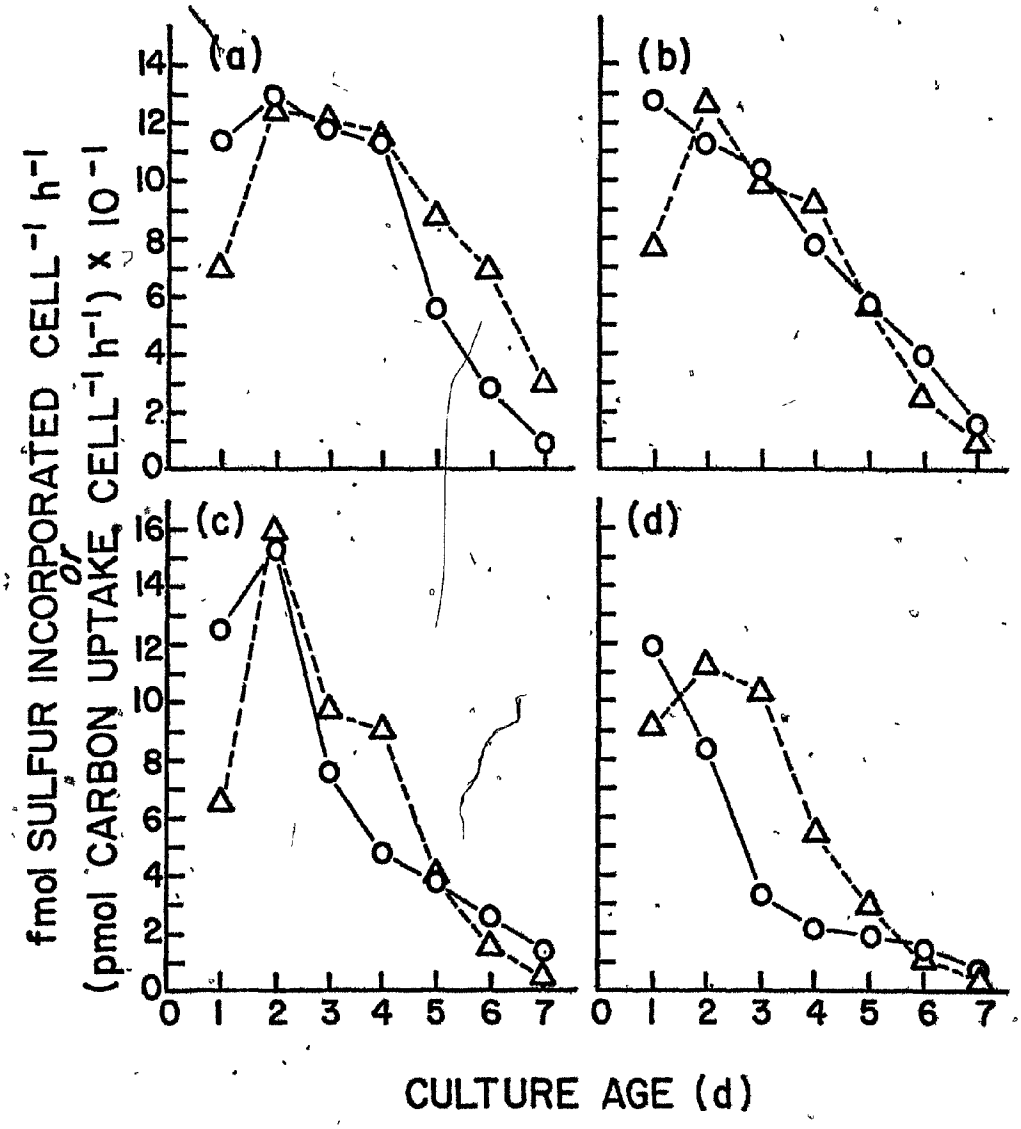

(a)
(b)
(c)
(d)
fmol SULFUR INCORPORATED CELL⁻¹ h⁻¹
or
(pmol CARBON UPTAKE CELL⁻¹ h⁻¹) x 10⁻¹
CULTURE AGE (d)

Figure 10. Growth of (a) T. weissflogii, (b) A. carteri, (c) D. tertiolecta, and (d) P. lutheri in batch culture with nitrate supplied at 18 µM. Biomass parameters are cell concentration (●) as (cell number ml^{-1}) X 10^4, chlorophyll a (■) as ng chl a ml^{-1}, and protein (▲) as µg protein ml^{-1}.

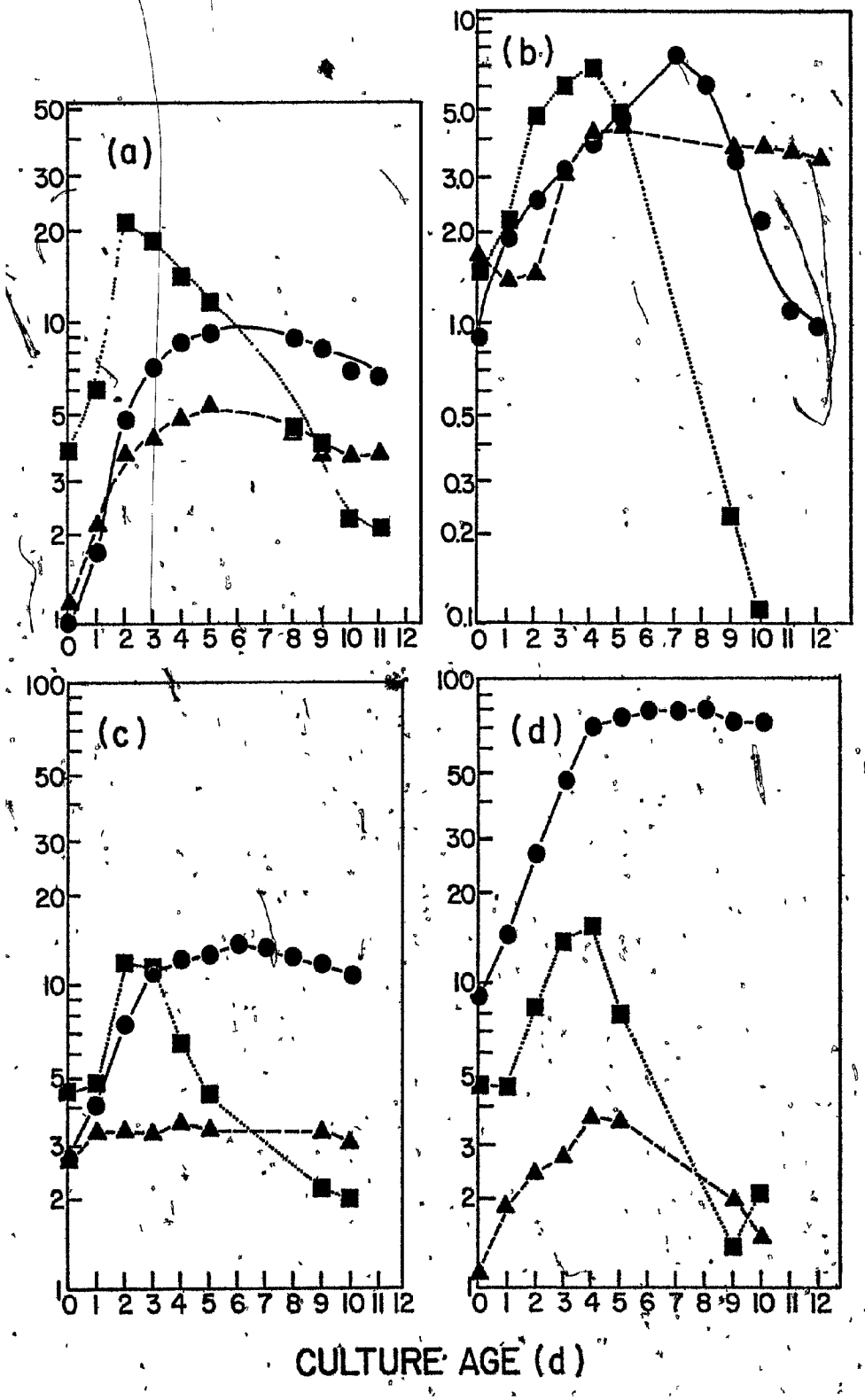

(a)
(b)
(c)
(d)
CULTURE AGE (d)

Figure 11. Rates of sulfur incorporation expressed on a unit protein basis by T. weissflogii (○), A. carteri (▲), D. tertiolecta (■), and P. lutheri (●) growing in batch culture with nitrate supplied at 18 µM.

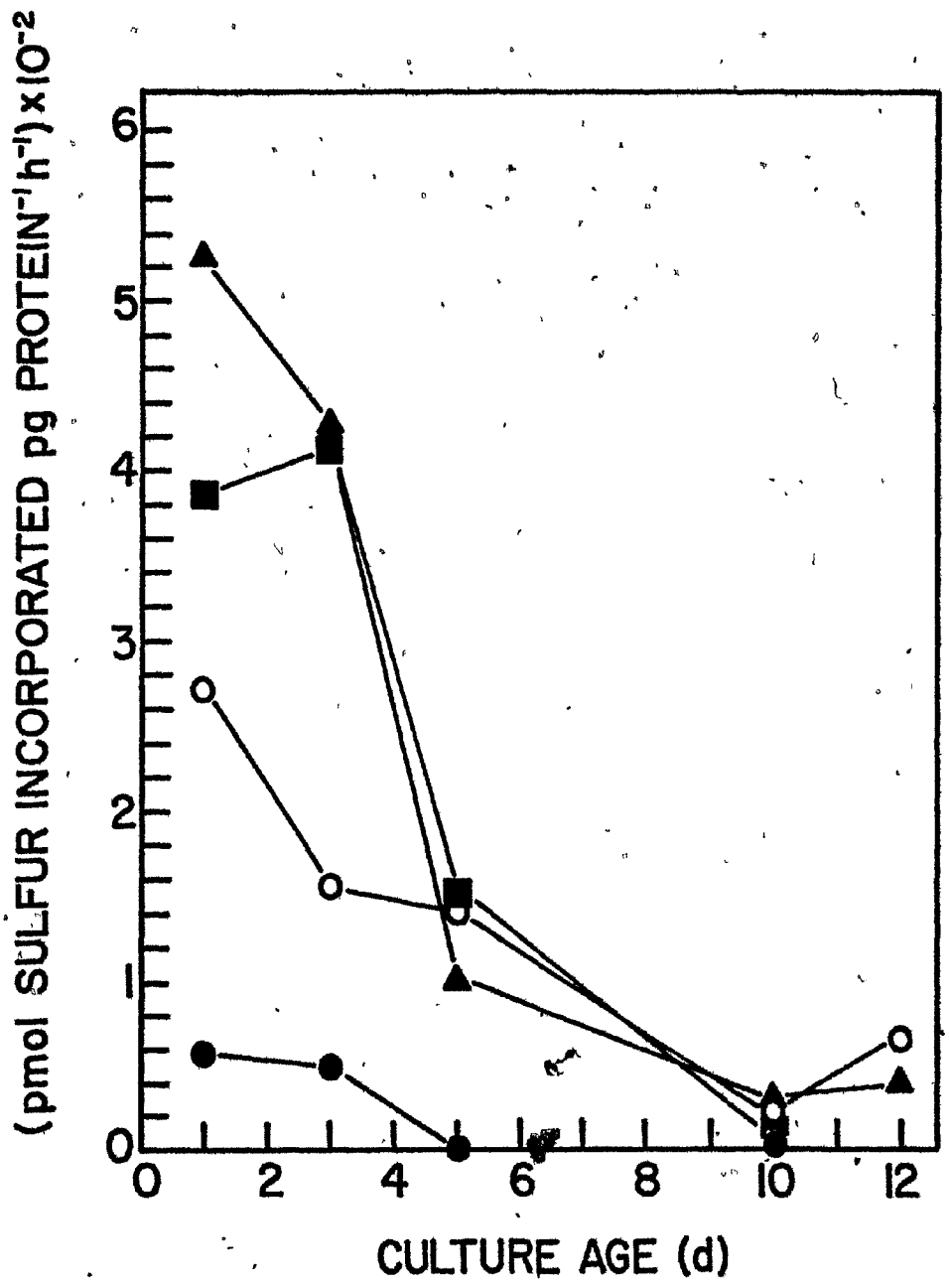
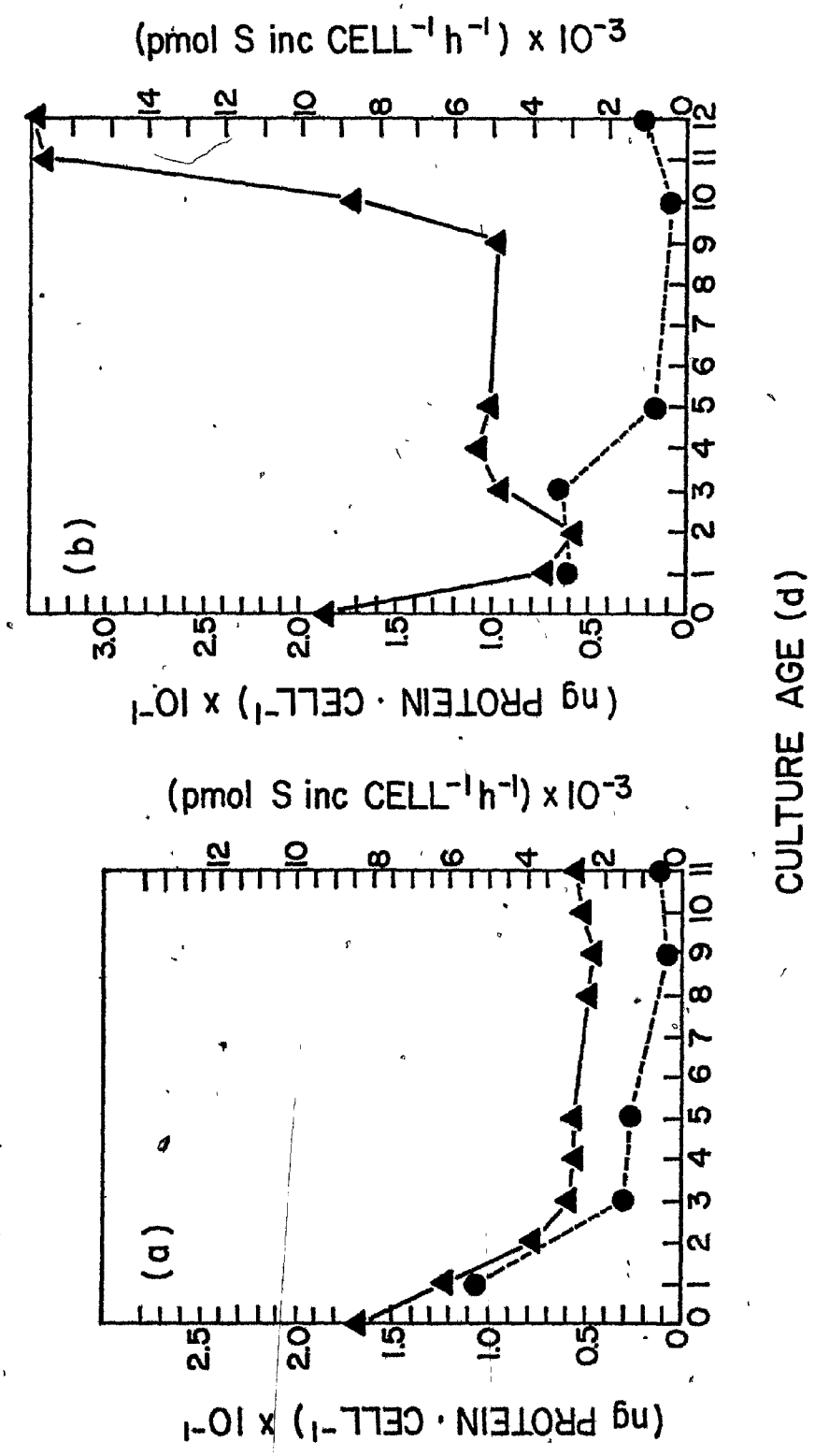
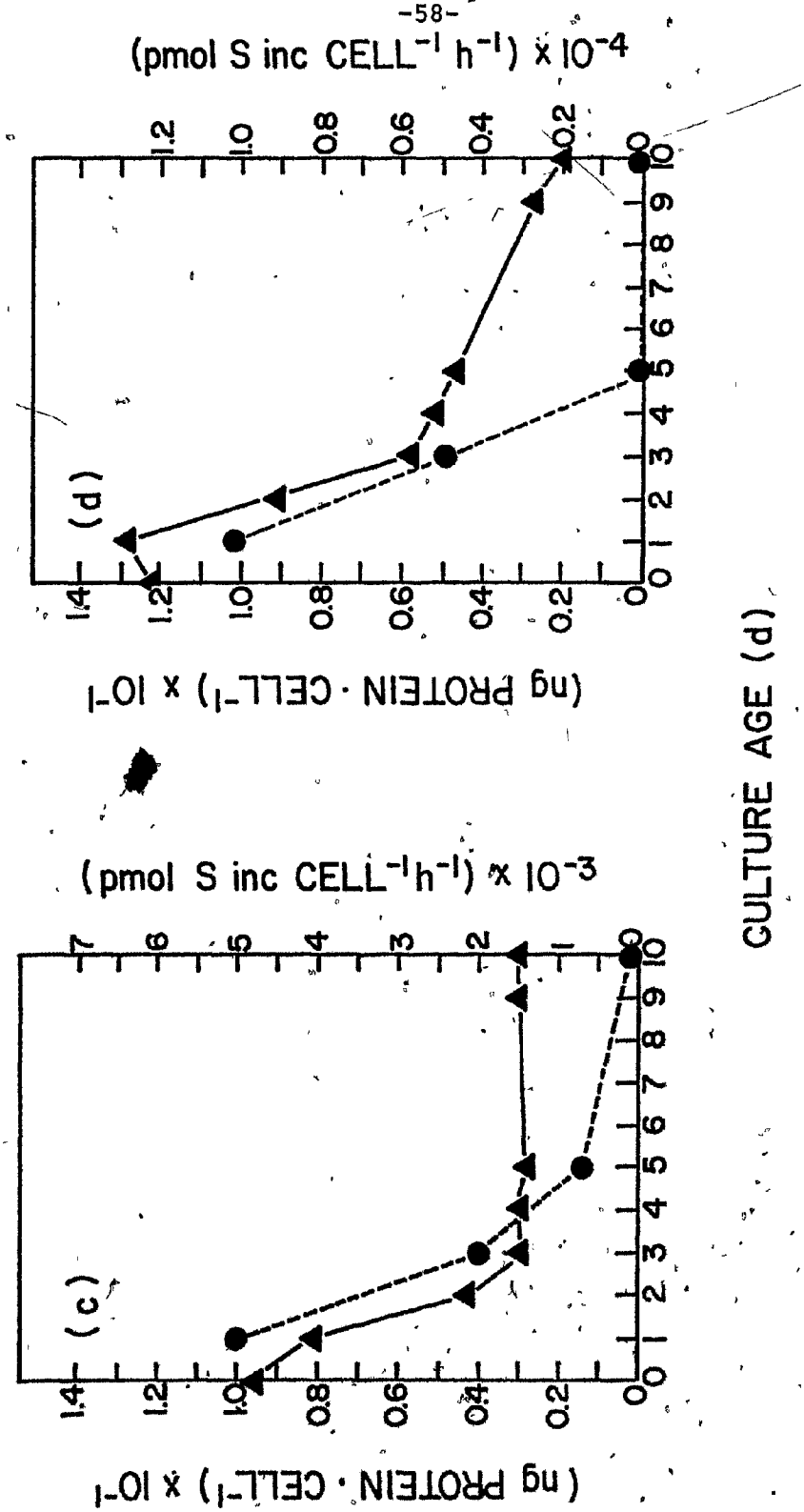

(pmol SULFUR INCORPORATED pg PROTEIN⁻¹h⁻¹) x10⁻²
0
1
2
3
4
5
6
0
2
4
6
8
10
12
CULTURE AGE (d)

Figure 12 (in two parts). Comparison between rates of sulfur incorporation (●), and accumulation of cellular protein (▲) by (a) T. weissflogii (b) A. carteri, (c) D. tertiolecta, and (d) P. lutheri growing in batch culture with nitrate supplied at 18 µM.


(pmol S inc CELL^{-1} h^{-1}) x 10^{-3}
(b)
(ng PROTEIN · CELL^{-1}) x 10^{-1}
(pmol S inc CELL^{-1} h^{-1}) x 10^{-3}
(a)
(ng PROTEIN · CELL^{-1}) x 10^{-1}
CULTURE AGE (d)


(pmol S inc CELL^{-1} h^{-1}) x 10^{-4}
(d)
(ng PROTEIN · CELL^{-1}) x 10^{-1}
(pmol S inc CELL^{-1}h^{-1}) x 10^{-3}
(c)
(ng PROTEIN · CELL^{-1}) x 10^{-1}
CULTURE AGE (d)

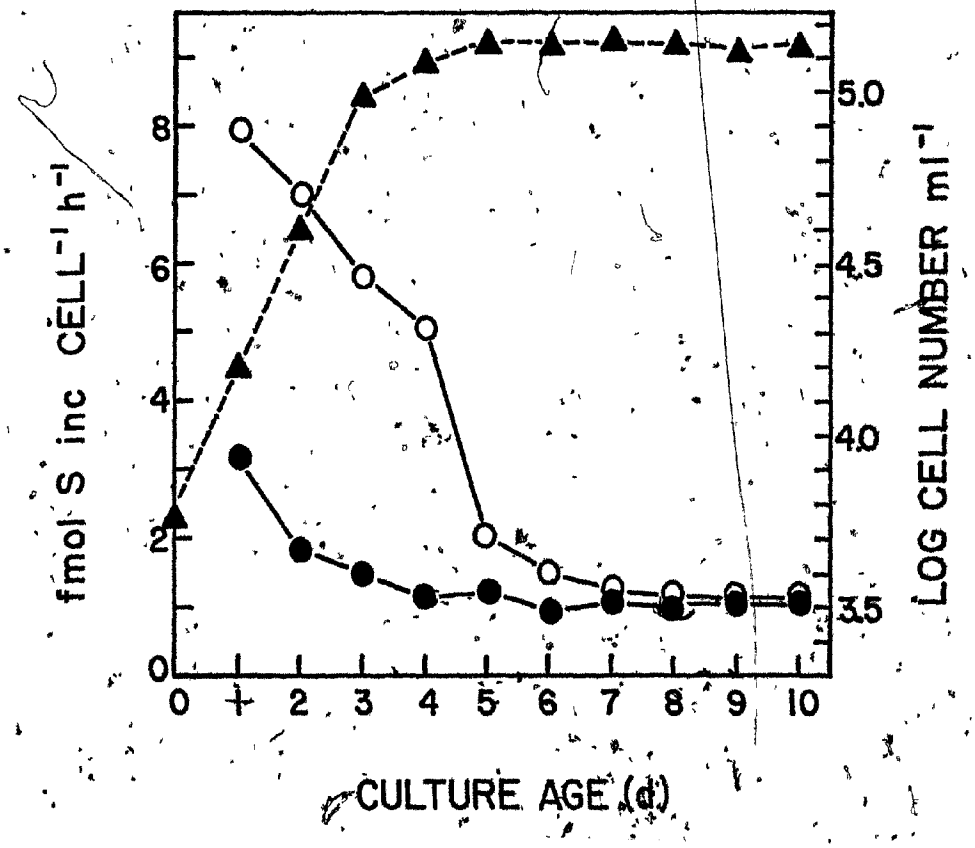
growing in f/2 were taken over the growth cycle and were incubated for 24 h in light and in darkness. The rate of ^{35}S incorporation (Fig. 13) and ^{35}S uptake per cell in the light declined gradually during the exponential phase, rapidly during the transition between exponential and stationary phases and reached a low but relatively constant value during the late stationary phase. The rate of ^{35}S incorporation in the dark was lower than in the light and declined gradually until the late stationary phase when the values in the dark and light were about equal (Fig. 13).

Chemostat culture. Rates of ^{35}S uptake and incorporation in the dark were obtained for T. weissflogii preconditioned in a nitrate-limited chemostat and are reported below in the section on "Ratios of rates of cellular processes".

Effect of nitrogen enrichment: Chemostat experiments

When a culture reaches stationary phase, growth may be affected by a number of factors (e.g., nutrients, light, pH, toxins). The particular factor encountered may greatly influence the way sulfur is utilized by the cell. Batch culture methods offer little control over growth limiting factors. Chemostat culture methods, however, allow one to control the growth limiting factor and to know the environmental history of the cell. I preconditioned T.

Figure 13. Log cell concentration (▲), and rates of sulfur incorporation per cell in light (○) and darkness (●) by T. weissflogii growing in batch culture with nitrate supplied at 883 µM.


fmol S inc CELL⁻¹ h⁻¹
LOG CELL NUMBER ml⁻¹
CULTURE AGE (d)
0
2
4
6
8
3.5
4.0
4.5
5.0
0 1 2 3 4 5 6 7 8 9 10

weissflogii in a N-limited chemostat and determined how sulfur and carbon are utilized as compared to batch culture conditions.

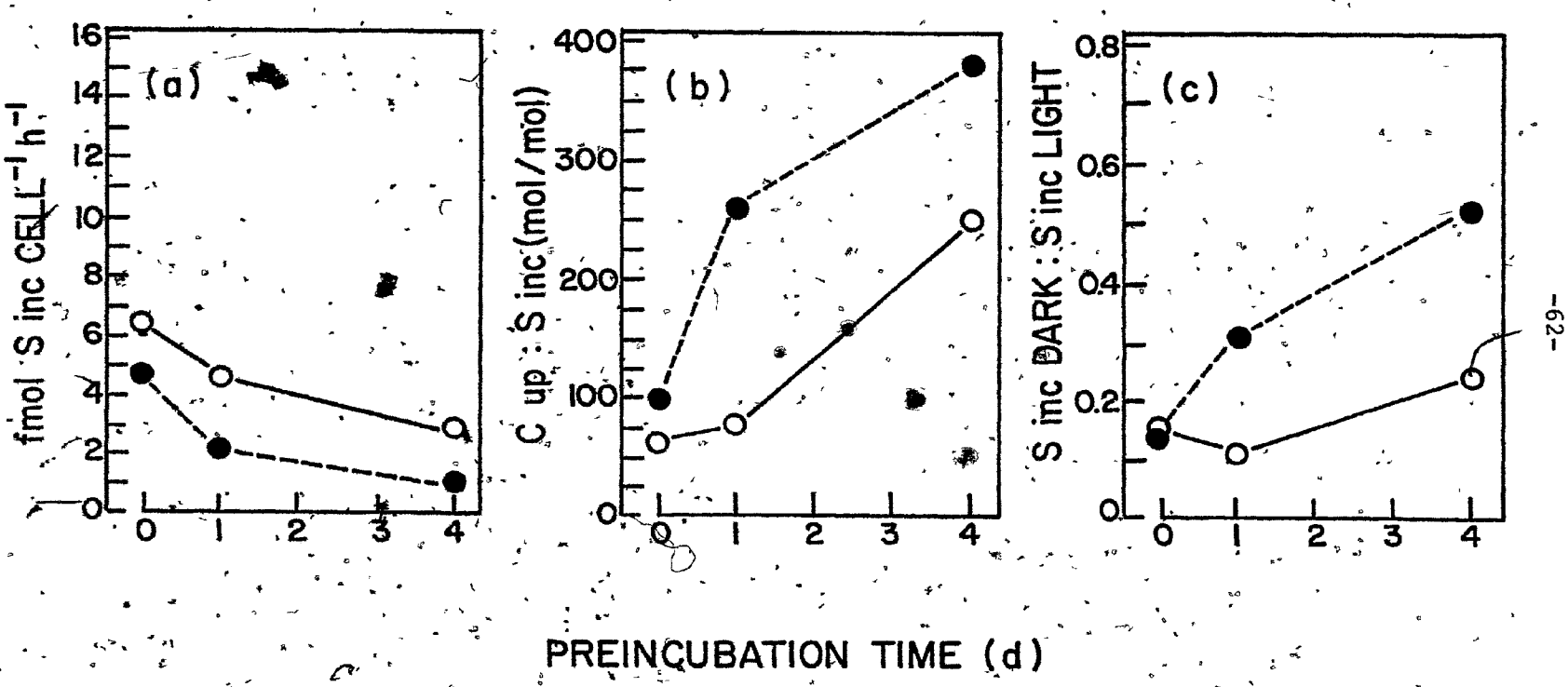
Rates of ^{35}S incorporation as well as cellular protein increased when nitrate was added to a N-limited steady-state culture (Table 4; Fig. 14a). In contrast, the rate of ^{14}C uptake was lower in the presence than in the absence of added nitrate (Table 4). Similar results were obtained with the addition of ammonium (not shown).

Rates of ^{35}S incorporation declined during a 4 d period after removal of the N-limited culture from the chemostat (Fig. 14a). Preincubation with 883 µM nitrate for 4 d also resulted in a decline in the rates, although they remained greater in the presence than in the absence of added nitrate (Fig. 14a). When the sample was removed from the chemostat culture system, it behaved essentially as a batch culture. The results obtained above are therefore analogous to those presented in Figs. 11 and 13 for batch cultures.

Ratios of rates of cellular processes

S incorporation : S uptake. Sulfur uptake and incorporation by T. weissflogii varied systematically over the growth cycle in batch culture (Fig. 13). The ratio of

Figure 14. Metabolic parameters for T. weissflogii withdrawn from a N-limited chemostat and incubated with (○) or without (●) 883 μM nitrate for 0, 1 or 4 d prior to measurement of sulfur incorporation, or photosynthetic carbon uptake. (a) Rate of sulfur incorporation per cell, (b) molar ratio of carbon uptake:sulfur incorporation, and (c) ratio of sulfur incorporation in the dark:sulfur incorporation in the light.


(a)
fmol S inc CELL^{-1} h^{-1}
16
14
12
10
8
6
4
2
0
0 1 2 3 4
(b)
C up : S inc (mol/mol)
400
300
200
100
0
0 1 2 3 4
(c)
S inc DARK : S inc LIGHT
0.8
0.6
0.4
0.2
0
0 1 2 3 4
PREINCUBATION TIME (d)

S inc : S up generally declined during exponential growth, increased during the early stationary phase, and then remained constant during the late stationary phase (Fig. 15). Addition of nitrate to a N-limited chemostat culture did not change the ratio in any consistent way.

The mean S inc : S up ratio for all experiments using T. weissflogii was 0.46 ± 0.10 S.D. (n = 43), and ranged between 0.32 and 0.58. Too few points were obtained over the growth cycle to define a trend in S inc : S up ratio for the three other species studied. The mean ratio was 0.53 ± 0.10 S.D. (n = 5) for A. carteri, 0.41 ± 0.05 S.D. (n = 4) for D. tertiolecta, and 0.16 ± 0.02 S.D. (n = 2) P. lutheri.

Carbon uptake : sulfur incorporation. The molar ratios of C up : S up and C up : S inc were low during exponential growth by T. weissflogii, elevated during the mid-stationary phase, and again low during the late stationary phase (Fig. 16). This general pattern varied slightly depending on the specific growth conditions and experiment. The ratio increased when the cells were grown in culture media containing low nitrate concentrations (not shown). Ratios for A. carteri were of the same order of magnitude as those for T. weissflogii and followed a similar pattern during growth in batch culture.

The C up : S inc molar ratio of T. weissflogii increased during a 4 d period after removal from a N-limited

Figure 15. Log cell concentration (▲) and ratio of sulfur incorporation:sulfur uptake (●) by T. weissflogii growing in batch culture with nitrate supplied at 883 µM.

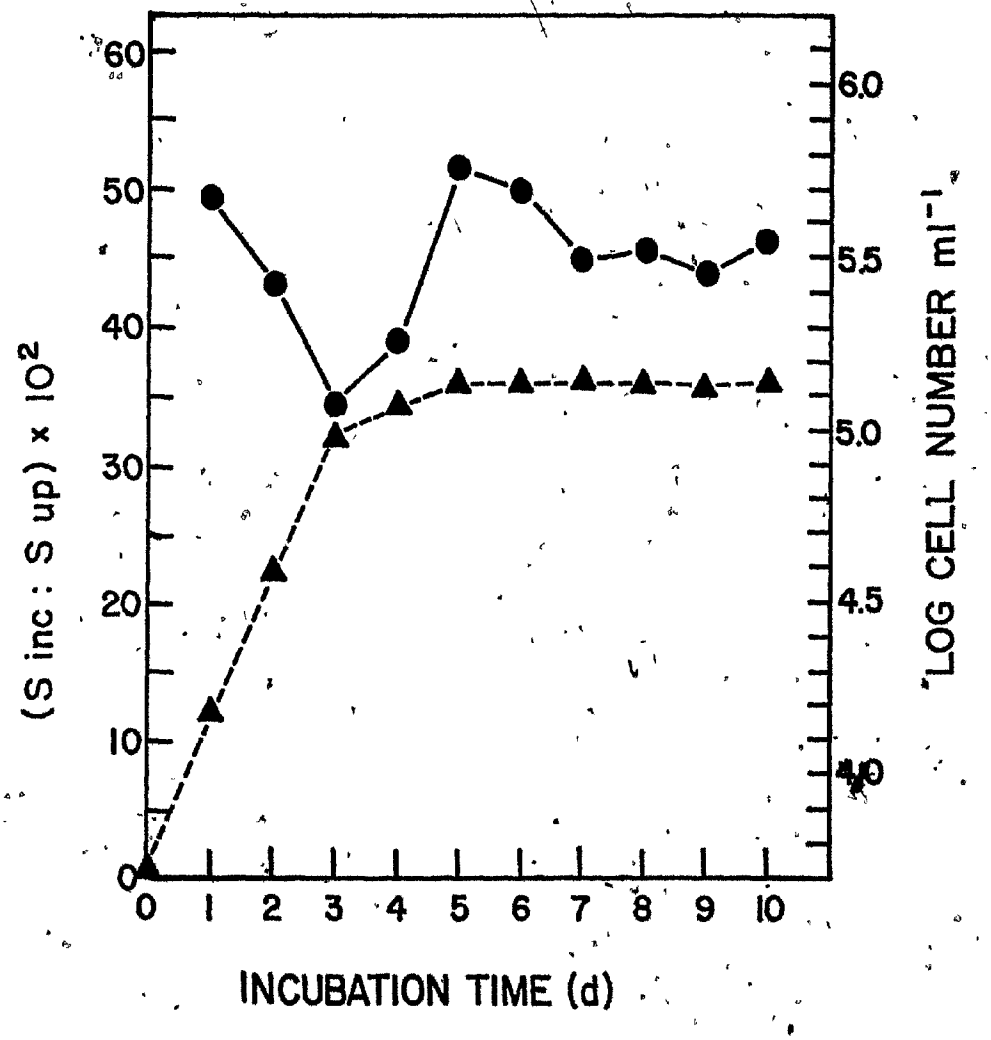
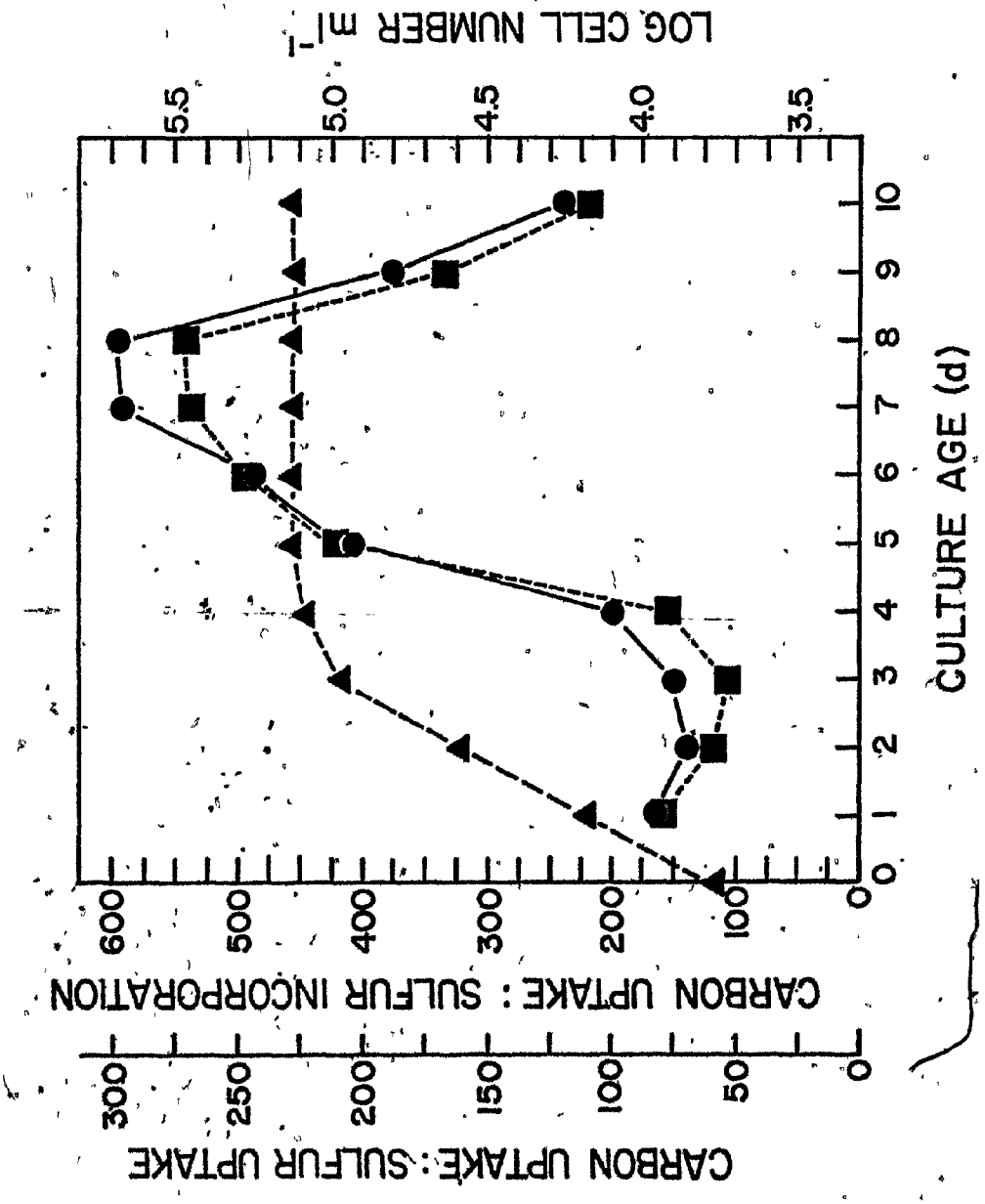

60
50
40
30
20
10
0
(S inc : S up) x 10^2
6.0
5.5
5.0
4.5
4.0
LOG CELL NUMBER ml^{-1}
0 1 2 3 4 5 6 7 8 9 10
INCUBATION TIME (d)

Figure 16. Log cell concentration (▲), molar ratio of carbon uptake:sulfur uptake (■) and molar ratio of carbon uptake:sulfur incorporation (●) by T. weissflogii growing in batch culture with nitrate supplied at 883 µM.


LOG CELL NUMBER ml⁻¹
5.5
5.0
4.5
4.0
3.5
CULTURE AGE (d)
0
1
2
3
4
5
6
7
8
9
10
CARBON UPTAKE : SULFUR INCORPORATION
600
500
400
300
200
100
0
CARBON UPTAKE : SULFUR UPTAKE
300
250
200
150
100
50
0

chemostat (Fig. 14b). Addition of nitrate at time-zero resulted in a constant ratio for the first 24 h of incubation followed by an increase during 4 d. The C up : S inc ratios remained significantly lower in the presence than absence of added nitrate (Fig. 14b).

Dark : light sulfur incorporation. During growth of T. weissflogii in batch culture (Fig. 13), the ratio of ^{35}S uptake or incorporation in the dark to ^{35}S uptake or incorporation in the light was low and constant during exponential growth, then rapidly approached 1.0 during the stationary phase (Fig. 17). There is an indication of a break in the slope during the mid-stationary phase which was repeatedly found in replicate experiments. A break in the slope of C up : S inc ratio vs. incubation time was also found on day 5 of the same experiment (Fig. 16).

The dark : light sulfur incorporation (Fig. 14c) and uptake ratios increased after removal of the culture from a N-limited chemostat. The low ratios found at time-zero and on day 1 in the presence of nitrate (Fig. 14c) are characteristic of exponentially growing cells (Fig. 17).

Inhibition of bacterial growth

Gentamycin, an inhibitor of bacterial protein synthesis (Caskey, 1973), prevented bacterial growth (Fig. 18a) and ^{35}S uptake (Fig. 18b) when supplied at greater

Figure 17. Log cell concentration (▲), ratio of sulfur uptake in the dark:sulfur uptake in the light (■), and ratio of sulfur incorporation in the dark:sulfur incorporation in the light (●) by T. weissflogii growing in batch culture with nitrate supplied at 883 µM.

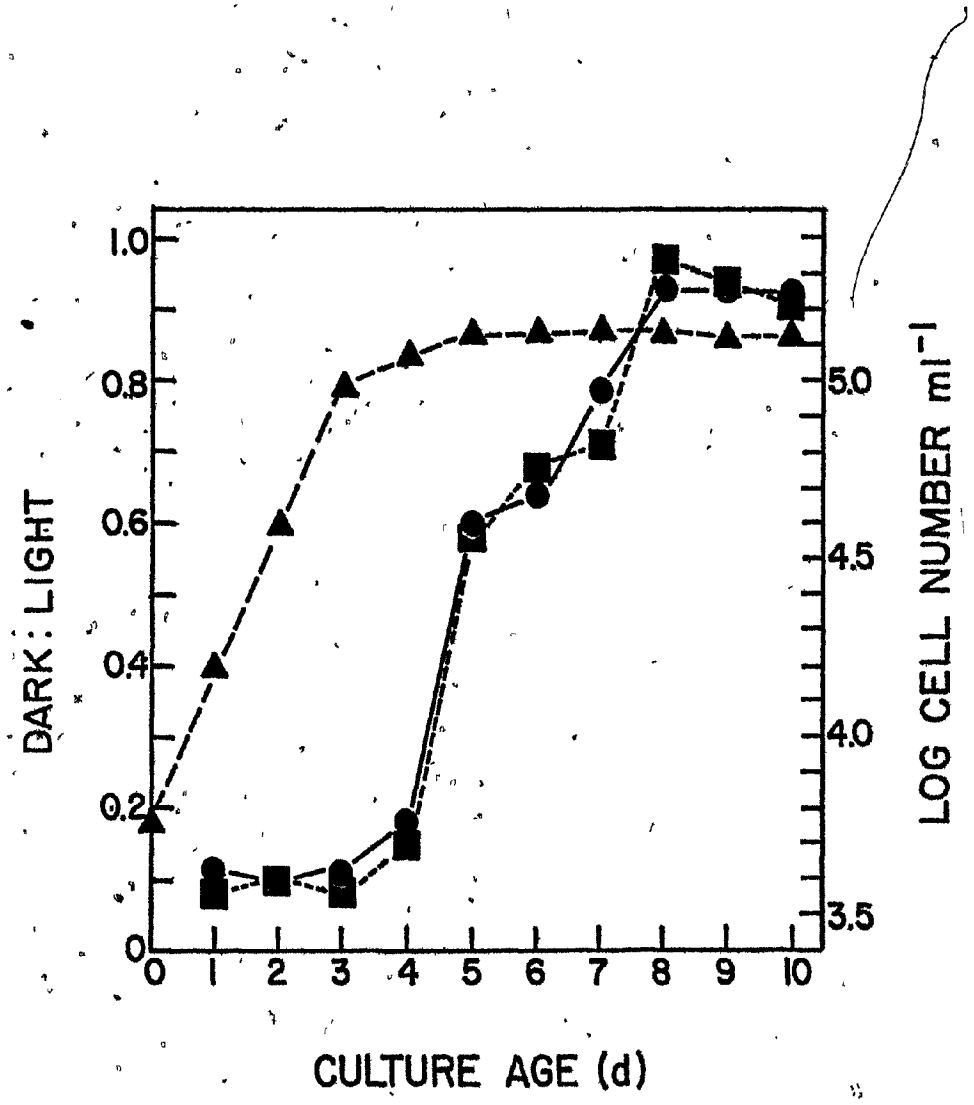
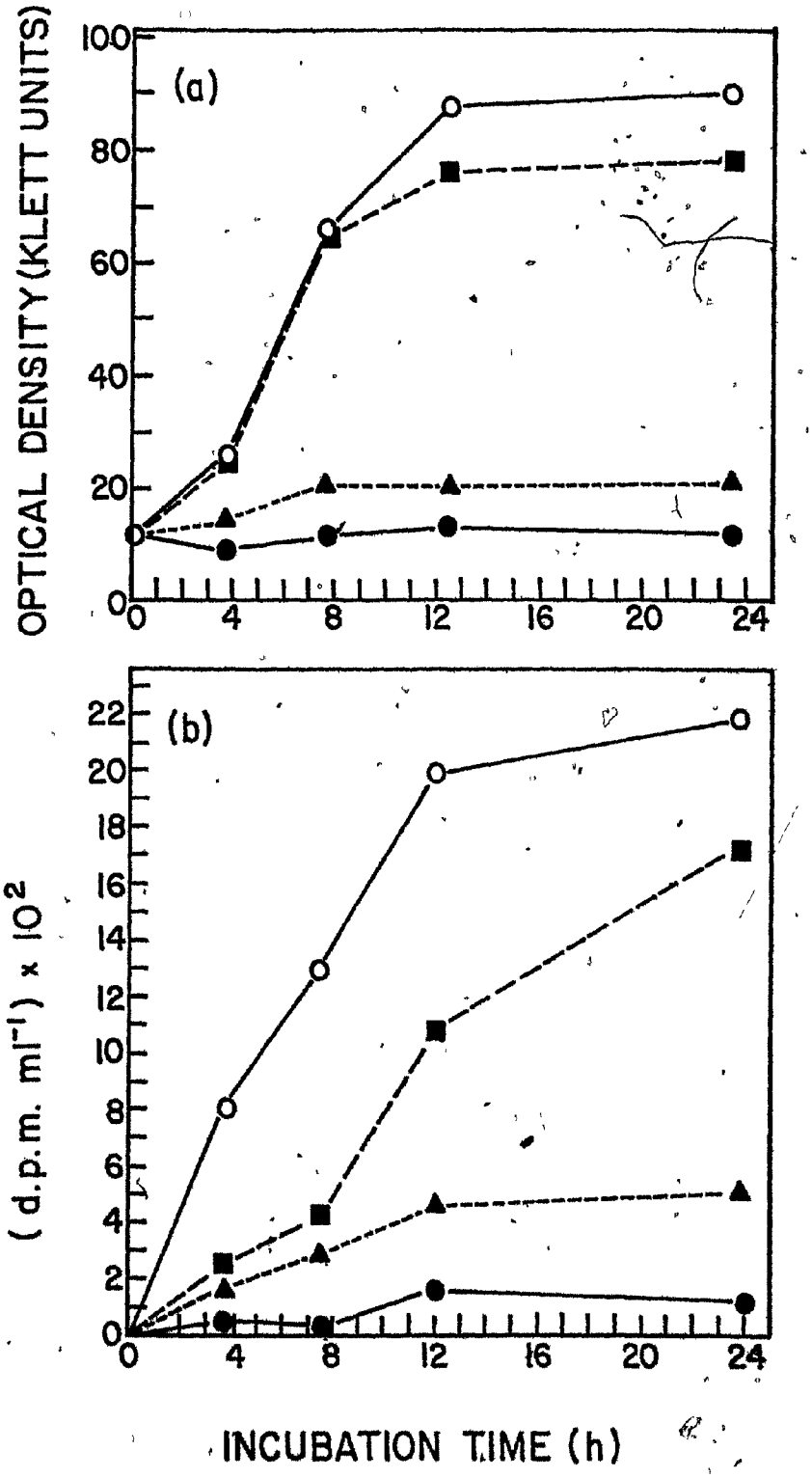

DARK : LIGHT
1.0
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10
CULTURE AGE (d)
LOG CELL NUMBER ml⁻¹
5.0
4.5
4.0
3.5

Figure 18. Growth (a) and ^{35}S uptake (b) by a mixed culture of marine bacteria in the dark and in the presence of 0 (○), 20 (■), 60 (▲), and 100 (●) µg gentamycin ml^{-1}.


(a)
OPTICAL DENSITY (KLETT UNITS)
100
80
60
40
20
0
0
4
8
12
16
20
24
(b)
(d.p.m. ml⁻¹) x 10²
22
20
18
16
14
12
10
8
6
4
2
0
INCUBATION TIME (h)

than 60 µg ml^{-1}. However, at greater than 60 µg ml^{-1}, it also stimulated ^{35}S incorporation (Fig. 19a) and ^{35}S uptake by T. weissflogii. A linear relationship was found between the percent stimulation of sulfur incorporation and gentamycin concentration at each incubation time (Table 6).

Sulfur-35 incorporation by a mixed T. weissflogii plus bacteria culture was significantly greater than by the alga alone at 3, 7 and 12 h of incubation (Fig. 19b). Addition of 60 µg gentamycin ml^{-1} did not reduce the ^{35}S activity during 12 h of incubation to the level of activity found by T. weissflogii alone, although 60 µg ml^{-1} was previously shown to inhibit bacterial growth and ^{35}S uptake when cultured separately (Fig. 18). After 24 h of incubation, however, the ^{35}S incorporated by the alga in the presence and absence of gentamycin and bacteria was the same (Fig. 19b).

Field Studies

North West Arm. Several experiments were carried out during the summers of 1976 and 1977, to test the feasibility of measuring ^{35}S incorporation in natural populations of marine microorganisms. Linear incorporation of ^{35}S was seen over a 12 h incubation period, with linearity usually extending to 24 h (Fig. 20). The ^{35}S incorporated per 150 ml of sample is significantly above background. Increases

Figure 19. Rate of ^{35}S incorporation (a) by T. weissflogii in the presence of 0 (○), 60 (■), 100 (▲), and 200 (●) µg gentamycin ml^{-1}, and (b) by T. weissflogii (○), T. weissflogii plus 60 µg gentamycin ml^{-1} (■), T. weissflogii plus a mixed culture of marine bacteria (▲), and T. weissflogii plus bacteria plus 60 µg gentamycin ml^{-1} (●). The dark period is indicated by a solid bar on the X axis.

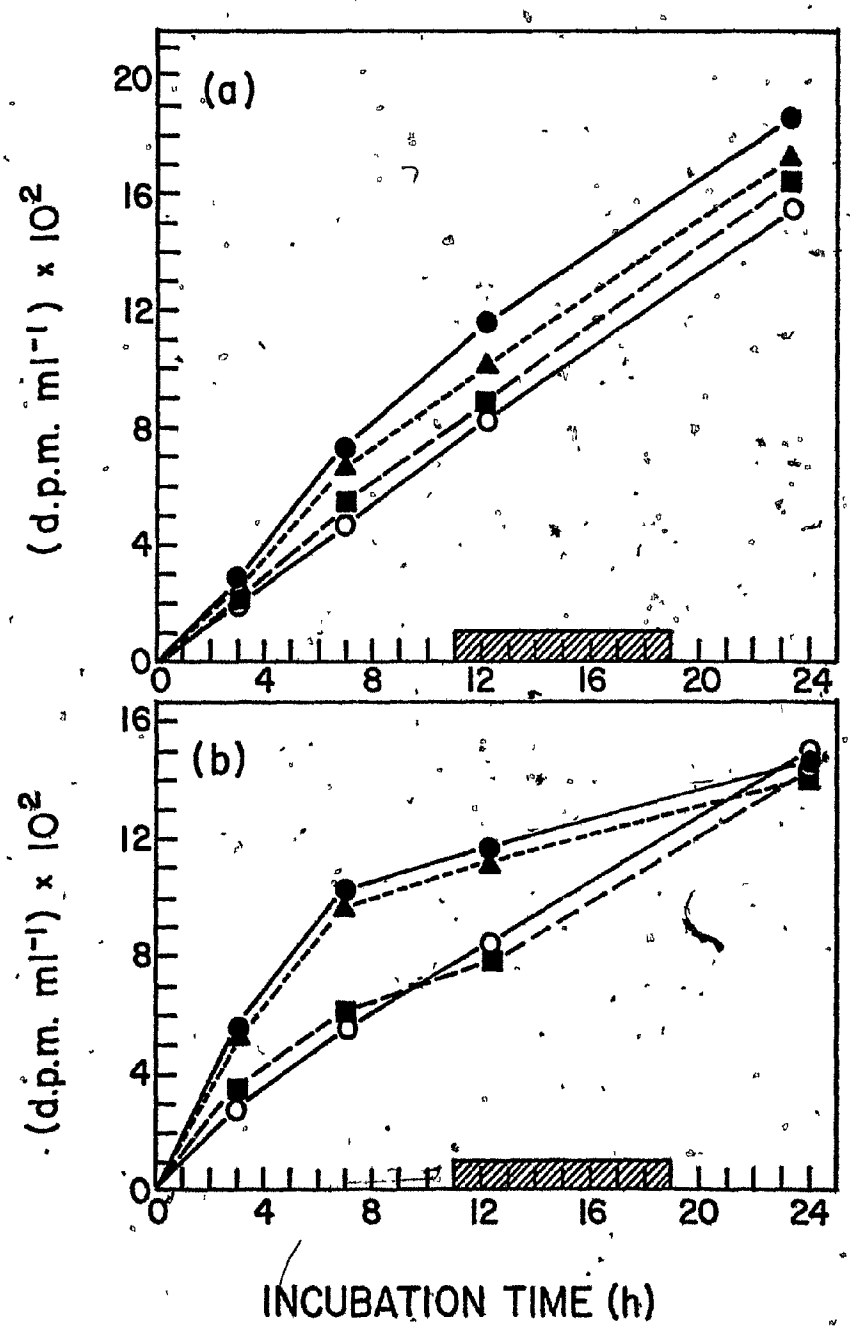
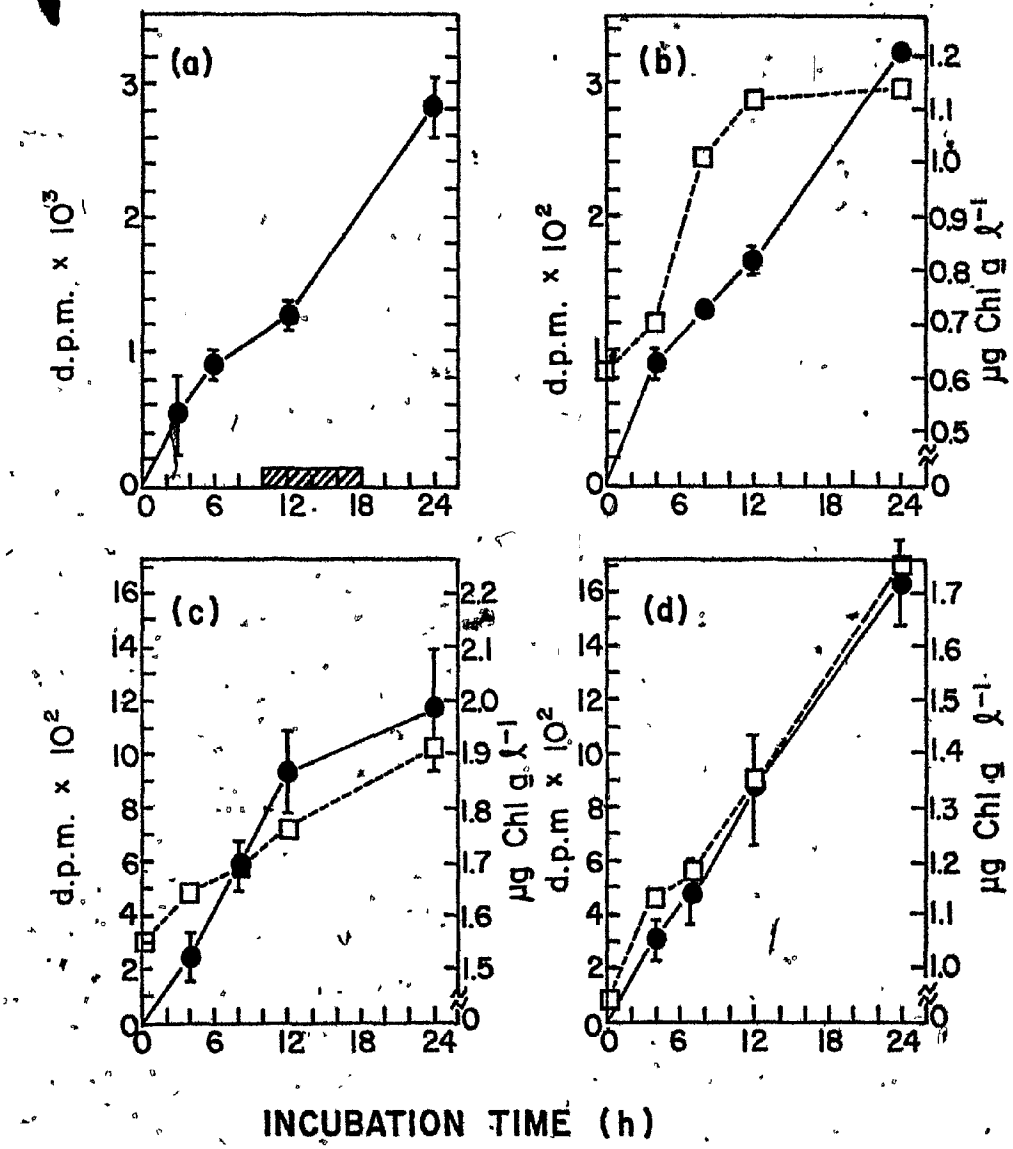

(a)
(b)
(d.p.m. ml⁻¹) x 10²
(d.p.m. ml⁻¹) x 10²
0
4
8
12
16
20
24
INCUBATION TIME (h)

Table 6. Percent stimulation of ^{35}S incorporation by T. weissflogii at 3, 7, 12, and 24 h of incubation due to the presence of 60, 100, and 200 µg gentamycin ml^{-1}. The data were obtained from Fig. 19a. Coefficients for the linear regression of % stimulation (y axis) on gentamycin concentration (x axis) are also shown.

Incubation time (h)	Gentamycin (µg ml^{-1})	Percent stimulation	m	b	r^2
3	60	3.4	0.26	-4.10	0.878
	100	33.8			
	200	45.2			
7	60	17.6	0.23	+9.84	0.907
	100	41.9			
	200	54.0			
12	60	8.5	0.22	-2.49	0.983
	100	23.2			
	200	41.2			
24	60	2.9	0.11	-2.20	0.973
	100	10.9			
	200	19.2			
All points	-	-	0.21	+0.26	0.726

Figure 20. Sulfur-35 incorporation per 150 ml of sample (●) and chlorophyll a concentration (□) of natural populations of microplankton from the North West Arm, Halifax, on (a) July 28, 1976, (b) August 26, 1977, (c) September 1, 1977, and (d) September 6, 1977. Experiments were conducted in continuous light except for (a) which was incubated with a 16:8 h light:dark cycle. Error bars indicate one standard deviation.


(a)
(b)
(c)
(d)
d.p.m. x 10^3
d.p.m. x 10^2
µg Chl g l^{-1}
INCUBATION TIME (h)

in chlorophyll a concentration and ^{14}C uptake with time generally paralleled increases in ^{35}S incorporation (Fig. 20; Table 7).

Molar ratios of S inc : chl a and C up : S inc for the natural populations (Table 7) are comparable to those of a T. weissflogii culture during the early stationary phase (Figs. 16, 25). The dark : light ^{35}S incorporation ratio (0.41) measured on September 1, 1977, is also comparable to that of an early stationary phase culture (Fig. 17). The dark : light ^{35}S incorporation ratio (0.08) obtained on July 28, 1976, is characteristic of an exponentially growing culture (Fig. 17). The variation among replicates of natural populations (± 16 - 36% S.D.) is greater than the ± 1 - 8% S.D. usually found with laboratory cultures.

Peru. The ^{35}S incorporation technique was applied to natural populations from the west coast of South America (Fig. 1). The problem of retention of radioactivity by filters, however, was not yet resolved when the samples were taken, and large and variable blank values were encountered. The data must be examined with this limitation in mind. Rates of ^{14}C incorporation are reported in a data report (Bedford Institute of Oceanography Data Report, in preparation).

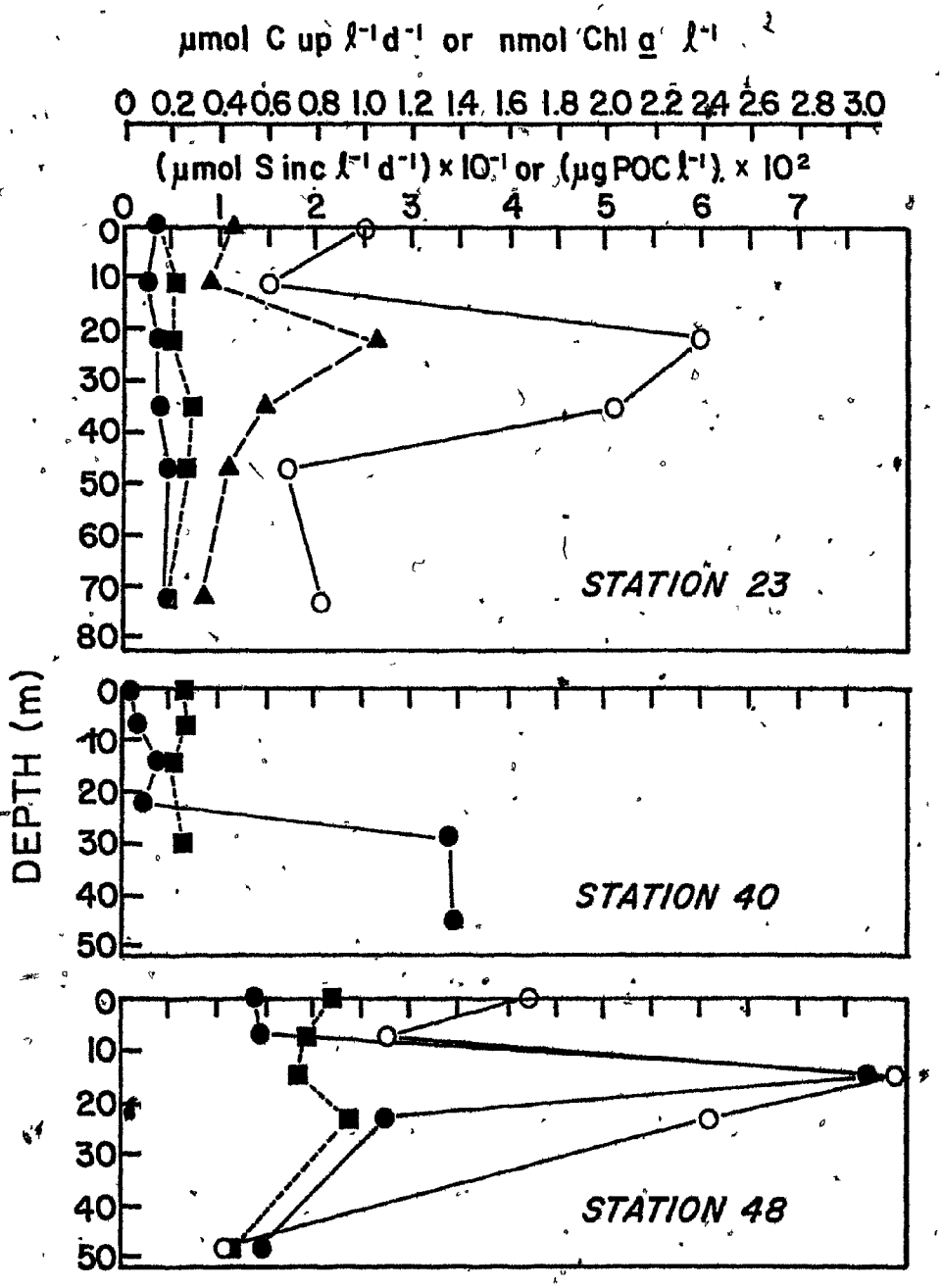
Rates of ^{35}S incorporation increased along a north to

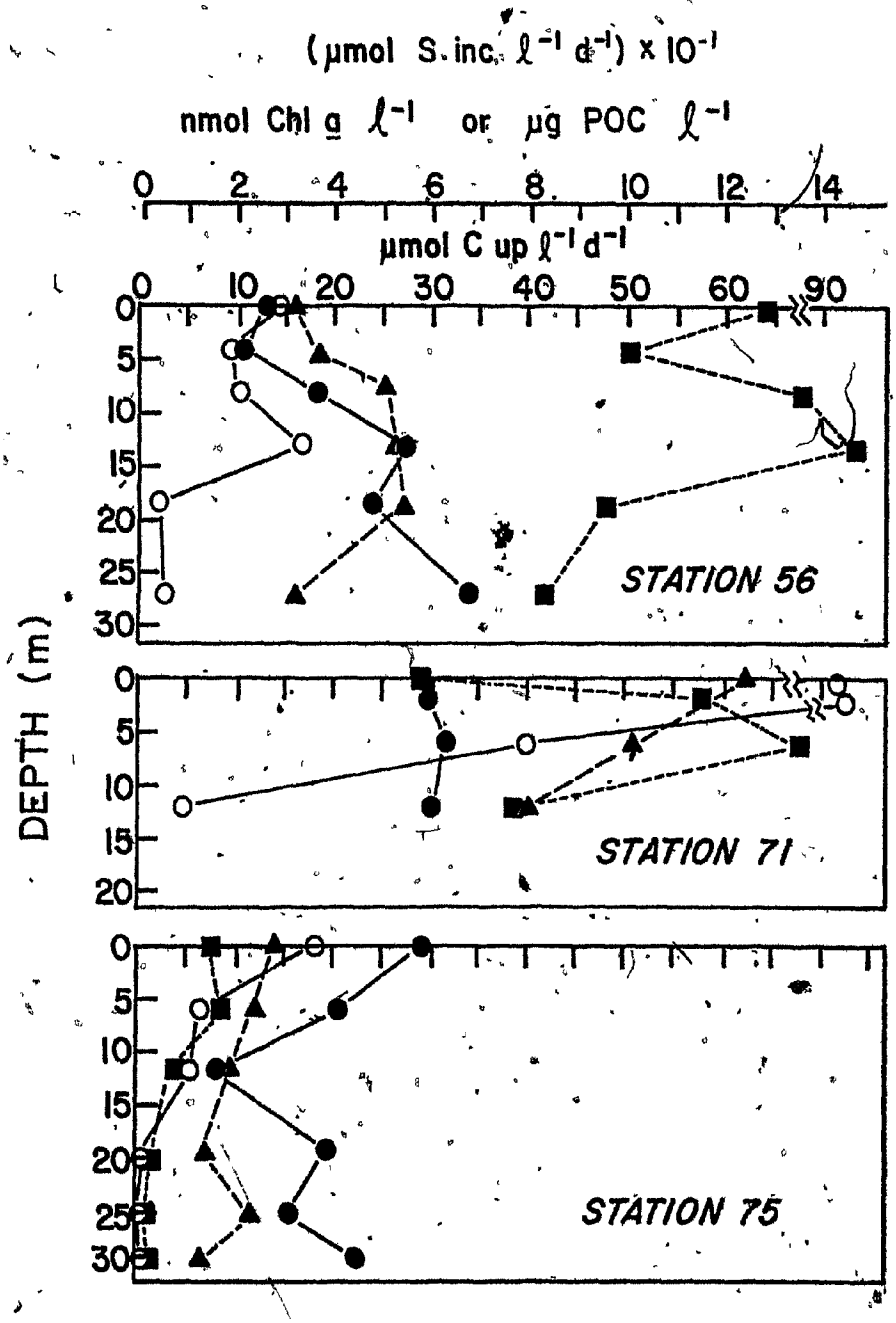
Table 7. Rates of sulfur incorporation, photosynthetic carbon uptake and chlorophyll a concentration (± S.D., n = 3) over a 24 h incubation period by natural populations of microplankton from the North West Arm, Halifax (1 mol chl a = 893.5 g chl a).

Date (d-mo-y)	Incubation time (h)	S inc (nmol ℓ^{-1} h^{-1})	C up (nmol ℓ^{-1} h^{-1})	Chl a (nmol ℓ^{-1})	C up / S inc (mol mol^{-1})	S inc / chl a (mol mol^{-1} h^{-1})
28-07-76	3.0	36.18 ± 25.26				
	6.0	29.32 ± 3.43				
	12.0	20.58 ± 1.56	n.d.	n.d.	n.d.	n.d.
	24.0	22.77 ± 1.87				
	24.0*	1.87* ± 0.03				
26-08-77	4.0	9.98 ± 2.81		0.78 ± 0.02		12.79
	8.0	7.17 ± 4.99	n.d.	1.13 ± 0.10	n.d.	6.35
	12.1	6.24 ± 2.18		1.25 ± 0.02		4.99
	24.0	5.93 ± 2.81		1.28 ± 0.09		4.63
01-09-77	4.1	10.92 ± 1.56		1.84 ± 0.10		5.93
	8.0	13.10 ± 2.18		1.89 0.01		6.93
	12.1	13.41 ± 3.43	n.d.	1.97 0.14	n.d.	6.81
	24.0	8.42 ± 2.18		2.14 0.04		3.93
	24.0*	3.43* ± 0.94		n.d.		n.d.
06-09-77	4.3	11.12 ± 2.50	1599 ± 52	1.26 ± 0.03	143.79	8.83
	7.3	10.29 ± 2.50	2057 ± 42	1.32 ± 0.09	199.90	7.80
	12.0	11.23 ± 2.81	1657 ± 42	1.50 ± 0.05	147.55	7.49
	24.0	10.60 ± 1.25	1196 ± 37	1.96 ± 0.07	112.83	5.41

* = incubation in darkness

Figure 21 (in two parts). Rates of sulfur incorporation (●), photosynthetic carbon uptake (○), chlorophyll *a* (■), and particulate organic carbon (▲) of microplankton from 6 stations offshore from the west coast of South America.


µmol C up ℓ⁻¹ d⁻¹ or nmol Chl a ℓ⁻¹
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0
(µmol S inc ℓ⁻¹ d⁻¹) x 10⁻¹ or (µg POC ℓ⁻¹) x 10²
0 1 2 3 4 5 6 7
DEPTH (m)
STATION 23
STATION 40
STATION 48


(µmol S.inc. ℓ⁻¹ d⁻¹) x 10⁻¹
nmol Chl a ℓ⁻¹ or µg POC ℓ⁻¹
0 2 4 6 8 10 12 14
µmol C up ℓ⁻¹ d⁻¹
0 10 20 30 40 50 60 90
DEPTH (m)
STATION 56
STATION 71
STATION 75

south transect parallel to the coast (Fig. 21). Highest values at all depths were found at Station 71 (Fig. 21), located 6.7 Km from the shore in an area of upwelling. A maximum in ^{35}S incorporation rate was generally found at depths corresponding to 11-25% of the surface light intensity. This maximum was roughly associated with maxima for chlorophyll a, POC, and ^{14}C uptake (Fig. 21). Positive correlations were found between sulfur incorporation rate and carbon uptake rate ($r = 0.685$, $n = 5$) for Station 48, POC ($4 = 0.802$, $n = 5$) for Station 56; and chlorophyll a concentration ($r = 0.589$, $n = 4$) for Station 71.

Molar ratios of S inc : chl a varied by an order of magnitude among depths at a given station, and two orders of magnitude among stations (Table 8). Values for the S inc : chl a ratio were generally within range found for T. weissflogii in batch culture (Fig. 25), and were similar to those obtained for natural populations from the North West Arm (Table 7). Molar ratios of C up : S inc (Table 8) were generally lower than those found in culture (Figs. 16, 24).

Davis Strait. Significant ^{35}S uptake above background was obtained at all stations (Table 9), although the cells were incubated with only about 0.3 µCi radiosulfate ml^{-1}. The cruise track was from south to north and

Table 8. Molar ratios of carbon uptake to sulfur incorporation and sulfur incorporation to chlorophyll a, and rate of cell division by natural populations of microplankton from the west coast of South America. Data for carbon uptake rate, sulfur incorporation rate and chlorophyll a concentration are found in Fig. 21.

Station number and date (d-mo)	Depth (m)	C up / S inc (mol mol^{-1})	S inc. / chl a (mol mol^{-1} h^{-1})	Growth rate (divisions d^{-1}) μ_1	μ_2	μ_3
23.	0	31.7	10.1	1.0	0.6	
	11	25.3	4.5	0.4	0.3	
(31-10)	22	71.0	7.1	1.4	0.4	0.1
	35	53.3	5.6	1.0	0.3	
	47	14.4	7.6	0.4	0.4	
	72	18.3	11.0	0.7	0.6	
40	1		1.4		0.1	
	7		2.0		0.2	
(01-11)	14		6.7		0.4	0.2
	22	n.d.	n.d.	n.d.	n.d.	
	29		56.7		2.9	
	45		n.d.		n.d	
48	0	12.4	6.5	0.3	0.4	
	7	7.7	6.7	0.2	0.4	
(02-11)	15	4.1	43.6	0.7	2.2	0.1
	23	8.8	12.1	0.4	0.7	
	48	3.0	13.5	0.1	0.7	
56	0	54.7	0.8	0.6	0.1	
	4	43.7	0.9	0.5	0.1	
(03-11)	8	27.4	1.1	0.4	0.1	0.1
	13	30.5	1.6	0.6	0.1	
	18	3.8	2.1	0.1	0.1	
	27	3.6	3.4	0.2	0.2	
71	0	156.9	4.2	1.6	0.3	
	2	155.6	2.1	1.0	0.2	0.1
(05-11)	6	62.8	2.0	0.5	0.1	
	12	7.8	3.3	0.1	0.2	0.1
75	0	31.2	16.3	1.4	0.9	
	6	16.1	9.6	0.6	0.5	
(06-11)	12	33.6	7.8	0.9	0.4	0.1
	19	2.0	54.9	0.4	2.8	
	25	1.6	109.3	0.6	5.6	
	29	1.1	63.6	0.3	3.3	

$\mu_1 = 3.32 \log [(C\ chl^{-1} + mg\ C\ mg\ chl^{-1}\ d^{-1})(C\ chl^{-1})^{-1}]$

C chl a^{-1} = 102 (sta. 23, 48, 71 and 75) and 30 (sta. 56)

$\mu_2 = 0.05\ (S\ inc\ chl^{-1}\ h^{-1}) + 0.04$ (see Fig. 25)

$\mu_3 = 0.06\ (S\ inc\ Dark : S\ inc\ Light)^{-1.12}$ (see Fig. 23)

Table 9. Sulfur uptake expressed as d.p.m. 250 ml^{-1} ± S.D. and nmol S $\ell^{-1}h^{-1}$ ± S.D., chlorophyll a concentration, and molar ratio of sulfur uptake rate to chlorophyll a by natural populations of microplankton from the Davis Strait (1 mol chl a = 893.5 g chl a).

Station number	Date (d-mo-y)	Experiment number	Incubation time (h)	Sulfur uptake (d.p.m. 250 ml^{-1})	Sulfur uptake (nmol S $\ell^{-1}h^{-1}$)	chl a (nmol ℓ^{-1})	S uptake / chl a (mol $mol^{-1}h^{-1}$)
46	19-04-78	G2	24.0	234.7 ± 42.2	3.34 ± 0.59	0.49	6.82
42	22-04-78	G4A	64.5	349.7 ± 8.1	2.40 ± 0.03	0.67	3.58
42	22-04-78	G4B	66.0	618.0 ± 39.5	4.09 ± 0.06	0.67	6.10
26	24-04-78	G5A	22.5	844.7 ± 91.2	20.43 ± 2.21	13.46	1.52
26	24-04-78	G5B	23.0	618.5 ± 77.1	14.63 ± 1.81	13.46	1.08
24	04-05-78	G6A	26.5	987.3 ± 31.7	30.84 ± 1.00	13.58	2.27
24	04-05-78	G6B	27.5	750.3 ± 98.7	28.13 ± 3.71	13.58	2.07
23	04-05-78	G7A	50.0	1625.0 ± 327.0	29.41 ± 5.93	7.12	4.13
23	04-05-78	G7B	51.0	1917.5 ± 471.6	32.50 ± 7.98	7.12	4.56

encountered a phytoplankton bloom estimated to be moving from north to south as shown by changes in chlorophyll *a* concentration. Low rates of ^{35}S uptake were associated with low chlorophyll *a* concentrations, and high rates with higher concentrations. The ratio of S up : chl *a*, however, was generally greater for the pre-bloom stations than for the bloom or post-bloom stations. These ratios are within the range of S inc : chl *a* ratios obtained during the Peru cruise (Table 8). The bloom samples were dominated by *Thalassiosira gravida* (M. Huntley, pers. comm.).

DISCUSSION

It is often desirable to have alternative approaches for examining complex or abstract problems. One approach is usually not suitable for all circumstances, while a combination of methods may provide new insights that were not previously attainable. It was with this premise in mind that research was carried out into determining the feasibility of using ^{35}S to assess the physiological condition of marine phytoplankton.

The development of a new technique offers the opportunity to carefully evaluate and test each step. Areas that were concentrated on during this study included resolution of technical problems associated with filtering ^{35}S-labelled phytoplankton, isolating cellular ^{35}S-protein, and measuring ^{35}S uptake and incorporation under a variety of environmental conditions.

A major technical difficulty encountered was the high blank caused by the retention of radioactivity by filters. This problem is common to ^{14}C (Morris et al., 1971a; Nalewajko and Lean, 1972; Williams et al., 1972; McMahon, 1973) and ^{35}S (Jordan et al., 1978; Campbell and Baker, 1978a; R. Monheimer, M. Jordan, pers. comm.). Filter blanks were reduced to an acceptable level by dialysis and prefiltration of the ^{35}S stock solution, by use of polycarbonate Nuclepore filters, and by careful rinsing of the

filter centre, edge and filter apparatus. Dialysis and pre-filtration of the ^{35}S stock solution eliminate possible radioactive macro-molecules and particulates. Nuclepore filters, by virtue of their thinness (10 μm), present less area for adsorption of radioactivity than cellulose nitrate membrane or glass fiber filters. Careful washing of the entire filter plus filter holder is necessary because the high specific activity of radiosulfate in the seawater may easily swamp the low percent (ca. 0.01%) of radioisotope taken up by the cells. Rinsing the filter edge with seawater removes radioactivity from an area of the filter that is covered by the lip of the filter chimney during the washing step.

Sulfur-35 incorporation as a tag for cellular protein

Several independent lines of evidence indicate that the measured ^{35}S activity, obtained by treatment of the cells with TCA and methanol:ether, is associated predominantly with the cellular protein fraction:

1) The pattern of ^{35}S incorporation by three species generally parallelled that of protein concentration over the growth cycle in batch culture (Fig. 12a,c,d),

2) Cycloheximide inhibited protein synthesis and blocked the incorporation of ^{35}S into the TCA plus methanol: ether insoluble fraction of T. weissflogii (Table 4).

3) Sulfur-35 was associated with the protein peak

obtained by Sephadex gel chromatography (Fig. 3a),

4) Hydrolysis of the protein fraction yielded ^{35}S associated with amino acids (Table 5). Hydrolysis also yielded ^{35}S which could not be definitely associated with amino acids. It is not likely that much of this uncharacterized material is sulfolipid or water-soluble polysaccharide, as these were removed in previous elution steps. It is possible that the activity represents ^{35}S-cystine which oxidizes to cysteic acid during acid hydrolysis at 110°C if dissolved air is present in the solution (Moore, 1963). Cysteic acid would pass through AG 50 columns without measurable retardation (Moore and Stein, 1951) and may appear in the water effluent (Giovanelli et al., 1978). The small amount of nitrogen (2.4% of that added to the column) found in the water eluate may be associated with the cysteic acid in that fraction. Nevertheless, if one considers the protein residue hydrolyzed, it accounted for about 50% of the total activity taken up by the cells, a value consistent with ^{35}S incorporation values usually determined (Table 2, Fig. 15).

Up to 20% of the measured ^{35}S in the TCA plus methanol:ether insoluble fraction, obtained by extracting the cells with solvents at room temperature while on the filter, is not associated with protein. This activity may represent sulfolipid-protein complexes (c.f., Haines, 1965) and could be removed by repeated extractions of the cells which were

first broken by homogenization or sonication (Table 3). Repeated extractions while the cells remained intact on the filter, however, did not remove significantly more activity (Table 2). The presence of this non-protein ^{35}S decreases the sensitivity of the method if one is interested in calculating rates of protein synthesis from ^{35}S data. However, it should not interfere when the method is used as an indicator of physiological state.

Sulfur-35 incorporation as an index of active biomass

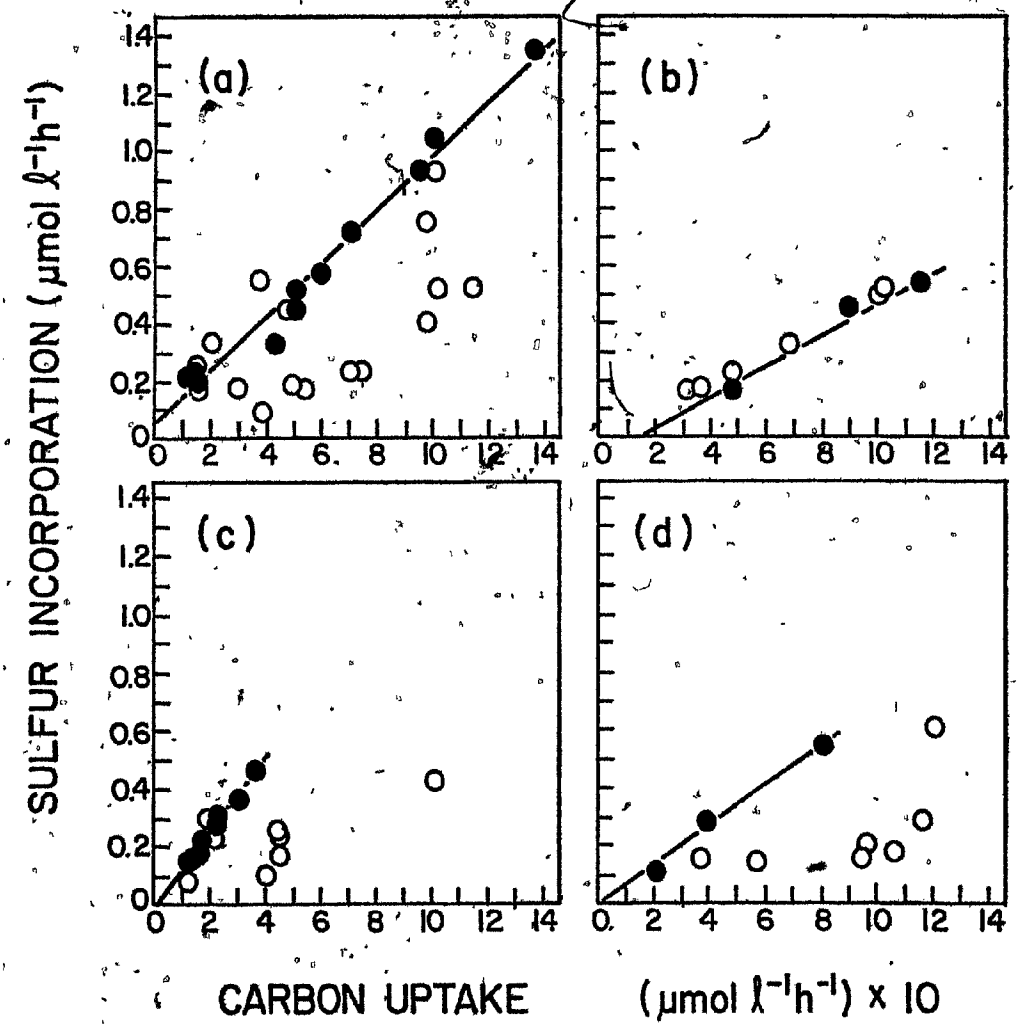
Measurement of ^{35}S incorporation is an excellent indicator of changes in phytoplankton biomass as shown by the high positive correlations between ^{35}S activity and cell number, in vivo fluorescence, chlorophyll a and protein concentration (Figs. 4a, 6). The positive x-intercepts for radioactivity vs. cell number and radioactivity vs. protein (Fig. 6c,d) are not significantly different ($p=0.01$) from the cell and protein concentrations, respectively, present in the medium at the time of inoculation with ^{35}S. The x-intercept therefore represents the amount of active biomass present at the start of an experiment. This method may be used in the field to determine what percent of the biomass is active. However, it appears that chlorophyll a or in vivo fluorescence may not be appropriate biomass parameters as positive x-intercepts were not obtained (Fig. 6a,b).

Sulfur-35 incorporation as an indicator of physiological state

The ^{35}S incorporation technique is a tool for assessing physiological state rather than biomass. In this respect, it must be shown that measurement of ^{35}S incorporation yields information that cannot be obtained by other methods. The rate of ^{35}S incorporation by T. weissflogii changes according to the growth stage in batch culture. The onset of senescence could generally be observed with ^{35}S at least 24 h before it was detected by changes in cell concentration (Fig. 7) or the rate of ^{14}C uptake (Fig. 8). During exponential growth, rates of ^{35}S incorporation and ^{14}C uptake were linearly related (Fig. 22a,b,d), resulting in a constant C up : S inc molar ratio. This suggests that the cells' requirements for carbon and sulfur are in balance during active growth when nitrogen is available for protein synthesis. The relationship also holds when nitrate is added to cells preconditioned in a N-limited chemostat (Fig. 22c). As a field tool, a constant C up : S inc molar ratio over an incubation period would indicate that the phytoplankton assemblage is growing actively (c.f. Fig. 16).

Unique information is given by measuring ^{35}S incorporation during the stationary phase when the rate of ^{14}C uptake generally exceeds that of ^{35}S incorporation

Figure 22. Regression of rate of sulfur incorporation (molar) on rate of carbon uptake (molar) by T. weissflogii growing in batch culture (a), (b), (d) and in chemostat culture (c). Data points are from the exponential (●) and stationary (○) phases of growth for batch culture, or from a N-limited chemostat culture supplemented with nitrate (●) and with nitrate absent (○). Linear regressions are for solid circles: (a) $y = 0.009x + 0.052$, $r^2 = 0.977$, (b) $y = 0.005x - 0.075$, $r^2 = 0.976$, (c) $y = 0.013x - 0.001$, $r^2 = 0.965$, and (d) $y = 0.007x + 0.002$, $r^2 = 0.991$.


(a)
(b)
(c)
(d)
SULFUR INCORPORATION (μmol ℓ⁻¹h⁻¹)
CARBON UPTAKE (μmol ℓ⁻¹h⁻¹) x 10
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
2
4
6
8
10
12
14

(Fig. 22a,d). This is to be expected if carbohydrate or lipid synthesis predominates over protein synthesis (e.g., Myklestad, 1977). Occasionally the rate of ^{35}S incorporation exceeds that of ^{14}C uptake when the cells are not actively growing. Some of these points represent the transition between the exponential and stationary growth phases, while others may be due to experimental error. In one experiment (Fig. 22b), carbon and sulfur were utilized in the same proporation during both exponential and stationary phases, although the experiment was conducted in the same manner as the others. There is also no clear explanation for why some points for the nitrogen-limited cells preconditioned in a chemostat lie on the same line as cells supplemented with nitrogen (Fig. 22b,c).

The inconsistencies in the relationship between ^{14}C uptake and ^{35}S incorporation render the C up : S inc molar ratio less than satisfactory as an index of physiological state. Furthermore, the ratio for T. weissflogii is generally low during both active growth and late senescence (Fig. 16), making it difficult to interpret low ratios. Monheimer (1978) found an analogous pattern in C:S uptake ratio by Nannochloris sp. and Chlorella sp., while Scenedesmus sp. showed a constant ratio during late senescence.

The ratio of S inc : S up was likewise not informative about the cell's physiological state. Although there was a

characteristic change in the ratio during the exponential and stationary phases (Fig. 15), a single value could not be used to distinguish between actively growing and senescent cells. Because rates of ^{35}S uptake and ^{35}S incorporation responded in a similar way to a given environmental condition (Figs. 14, 16, 17), and because the former is easier to measure than the latter, information about physiological state could just as well be obtained by measuring ^{35}S uptake as by ^{35}S incorporation. A S inc : S up ratio of 0.5, averaged for all experiments, could be used to convert ^{35}S uptake to ^{35}S incorporation.

The ratio of ^{35}S uptake in the dark to ^{35}S uptake in the light is a promising index of physiological state. This ratio was low and constant during the exponential phase of T. weissflogii growth, and elevated during senescence (Fig. 17). The ratio responded in a similar way for T. weissflogii grown in a chemostat (Fig. 14e,f). If the dark:light ^{35}S uptake ratio changes accordingly in other species of phytoplankton, then the ratio may become a useful field tool.

Under some circumstances, the dark:light ^{35}S uptake ratio can be used to ascertain the growth history of a phytoplankton population as well as to predict its future development. An assemblage in active growth, as determined by a low dark:light ^{35}S uptake ratio, can be expected to

have been that way and continue to remain so for an unspecified yet limited time. Less predictive value is possible if a population is maintained in active, steady-state growth by constant nutrient input and loss terms.

One may speculate as to whether the relationship between the dark:light ^{35}S uptake ratio and physiological state is characteristic of other species of phytoplankton. There are no known reports in the literature of this relationship per se, however, values are available for the uptake rate of sulfate in the dark. Uptake in the dark was about 19% of that in the light for Chlorella (Wedding and Black, 1960), 80% for Pavlova lutheri (Deane and O'Brien, 1975), 30-50% for the macroalga Fucus serratus (Coughlan, 1977), 25% for Anacystis nidulans (Jeanjean and Broda, 1977), and 70% for Navicula pelliculosa (Campbell and Baker, 1978b). The physiological condition of these algae at the time of measurement of sulfate uptake in the dark is not always made clear. Monheimer (1978) found that "dark sulfate uptake rates were closest to light sulfate uptake rates when the cells were reproducing most slowly", with reference to Nannochloris sp., Chlorella sp. and Scenedesmus sp. Jassby (1975), however, found negligible uptake of sulfate in the dark by Chlorella vulgaris and Scenedesmus obliquus, and Utkilen et al. (1976) reported similar results for Anacystis nidulans. Some of these

contradictory results may be due to differing physiological conditions of the cells. Nevertheless, the literature shows a general ability for algae to take up sulfate in the dark.

If it can be shown that there is a general similarity in energy sources for sulfate uptake by algae, then it may be inferred that there is also a common relationship between the dark:light ^{35}S uptake ratio and physiological state. Studies with CCCP, an inhibitor of both oxidative phosphorylation and photophosphorylation, and DCMU, an uncoupler of photosystems I and II, demonstrate that either photosynthetic or respiratory energy can support sulfate uptake in a wide variety of algae (Wedding and Black, 1960; Deane and O'Brien, 1975; Jeanjean and Broda, 1977; Coughlan, 1977). In the light, DCMU inhibits sulfate uptake to about the level of that found in the dark (Deane and O'Brien, 1975; Coughlan, 1977). Nutrient deficiency, which impairs the photosynthetic capacity of the cell and slows the flow of energy derived via photophosphorylation, may be considered to have an effect analogous to that of DCMU on sulfate uptake in the light. This is suggested by the rapid decline in the rate of ^{35}S incorporation (Fig. 13) and ^{35}S uptake with culture age to the level of that found in the dark. The decline in the dark was less dramatic (Fig. 13), suggesting that energy from oxidative

phosphorylation remains available for sulfur incorporation during the stationary phase.

Sulfur-35 incorporation as a measure of division rate

The change in the rate of ^{35}S incorporation by T. weissflogii with culture age (Figs. 16, 17) suggests that there may be a relationship between ^{35}S incorporation and division rate. Such relationships are shown for division rate vs. dark:light ^{35}S incorporation ratio (Fig. 23), C up : S inc (Fig. 24), S inc : chl a (Fig. 25), and S inc : protein (Fig. 26). Division rate was calculated from daily changes in cell number. Individual experiments are shown separately in Figs. 24-26 to illustrate similarities between batch and chemostat culture experiments. There is much scatter in the data, but power functions of the type $y = ax^b$ are suggested for relationships between division rate and dark:light sulfur incorporation ratio (Fig. 23) or division rate and C up : S inc ratio (Fig. 24), while linear functions may adequately describe the remaining relationships (Figs. 25, 26). Because of the scatter, it appears that a rectangular hyperbola of the type shown by Caperon and Meyer (1972) could also be fitted to the division rate vs. S inc : chl a relationship (Fig. 25a) where the data is most complete. There is more biological justification in choosing a hyperbolic rather than a linear function, as division rate cannot be expected to increase indefinitely.

Figure 23. Division rate of T. weissflogii as a function of ratio of sulfur incorporation in the dark:sulfur incorporation in the light. Data points are taken from batch culture (●) and chemostat culture (▲) experiments. A power function of the type $y = ax^b$ was fitted to the points such that divisions $d^{-1} = 0.06$ (S inc Dark:S inc Light)$^{-1.12}$, and $r^2 = 0.725$.

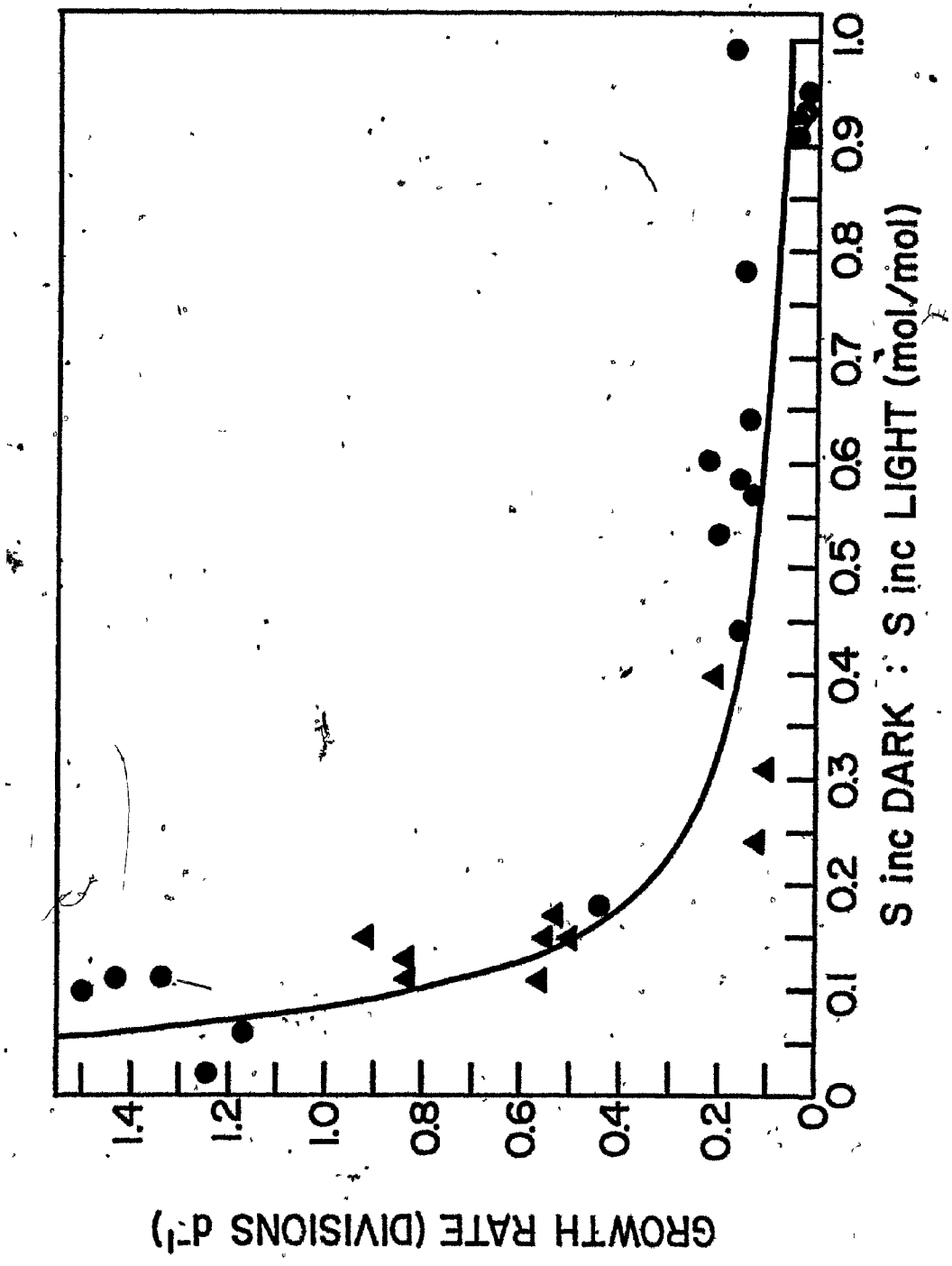

GROWTH RATE (DIVISIONS d⁻¹)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
S inc DARK : S inc LIGHT (mol/mol)

Figure 24. Regression of division rate of T. weissflogii on molar ratio of carbon uptake:sulfur incorporation for batch culture (a), (d), chemostat culture (c), and combined data from batch and chemostat culture experiments (b). Symbols as in Fig. 22.

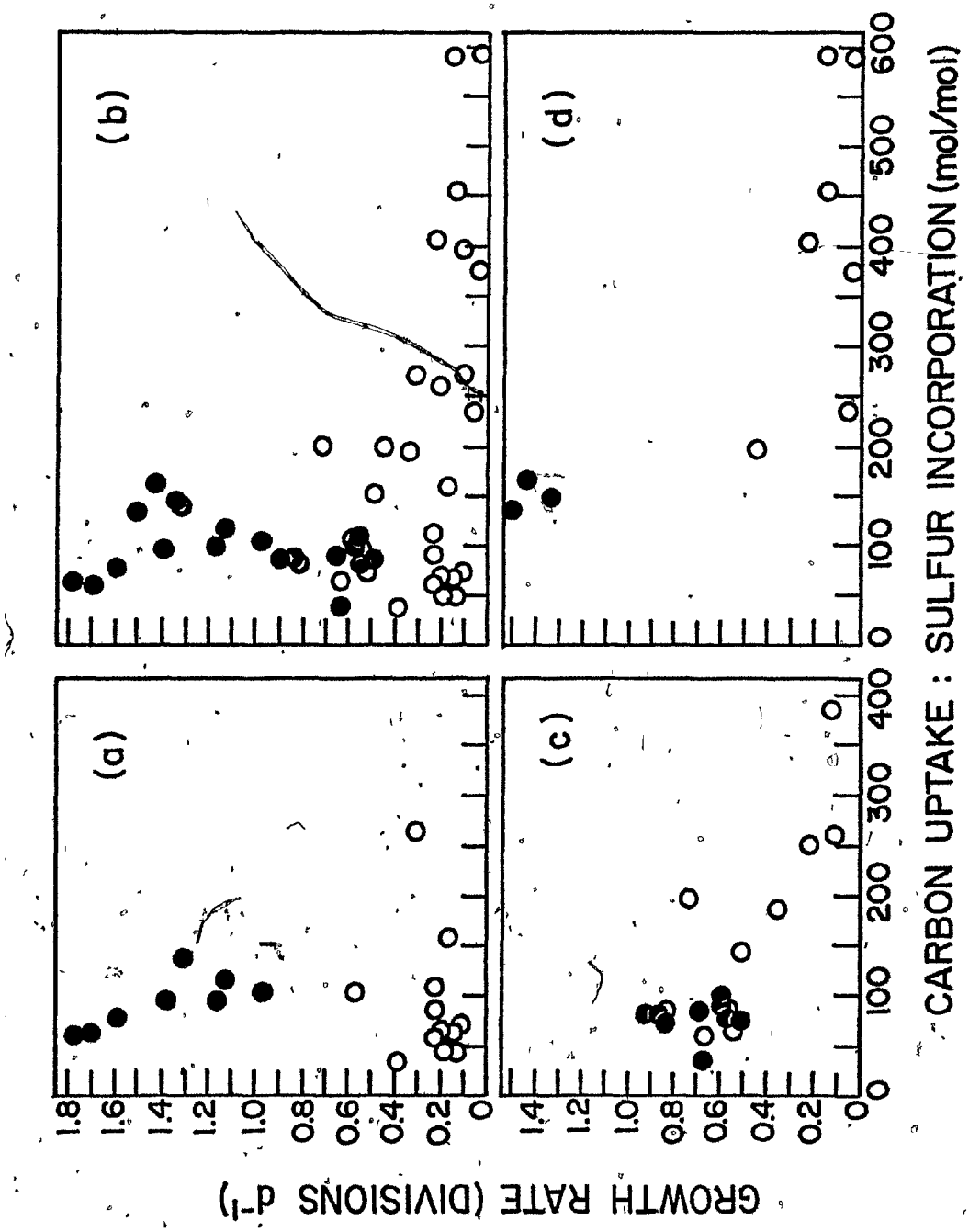

(a)
(b)
(c)
(d)
GROWTH RATE (DIVISIONS d⁻¹)
CARBON UPTAKE : SULFUR INCORPORATION (mol/mol)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
100
200
300
400
500
600

Figure 25. Regression of division rate of T. weissflogii on molar ratio of sulfur incorporation:chlorophyll a for batch culture (a), (d), chemostat culture (c), and combined data from batch and chemostat culture experiments (b). Symbols as in Fig. 22. The linear regression equation for (b) is $y = 0.05x + 0.04$, $r^2 = 0.636$.

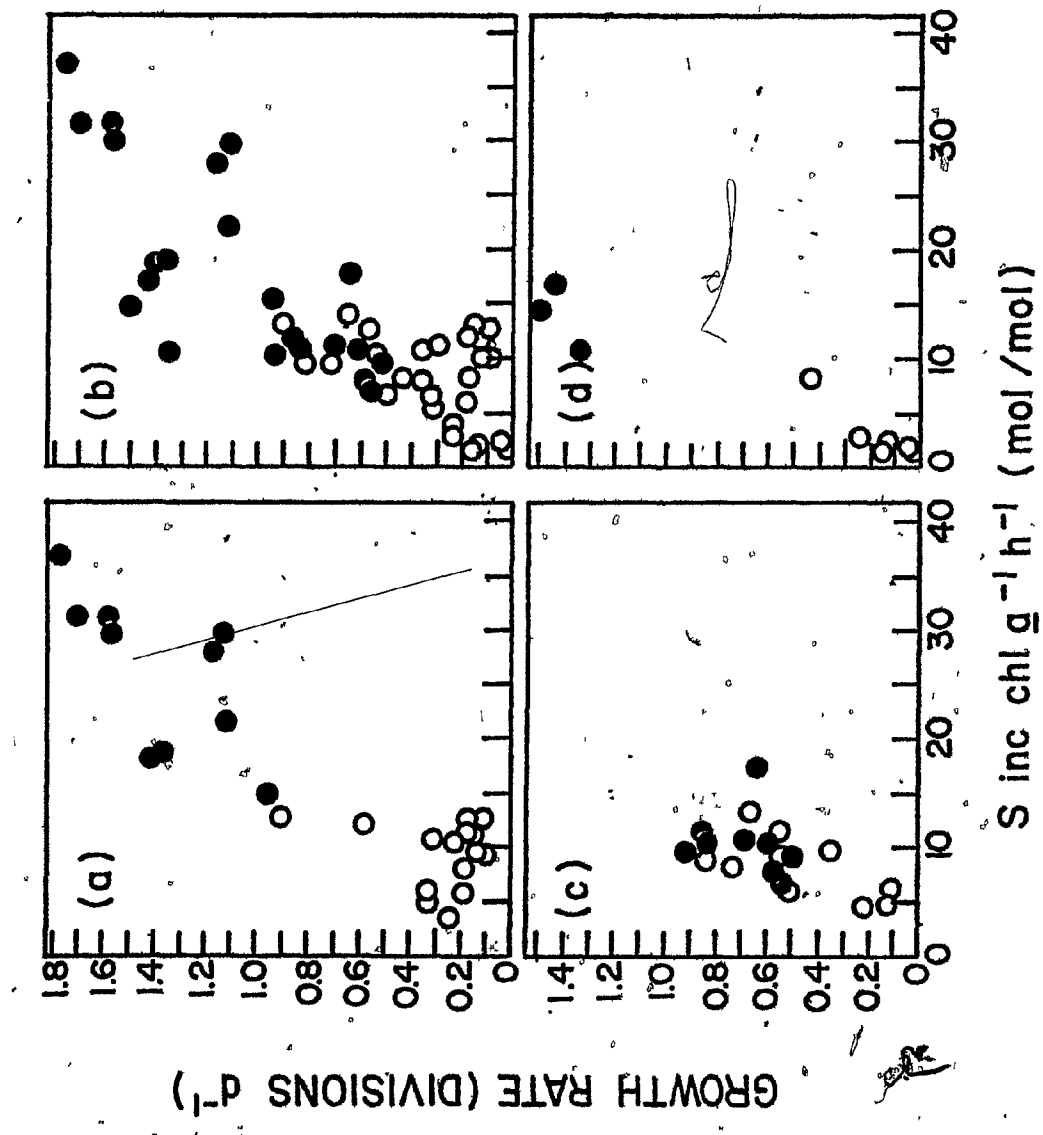
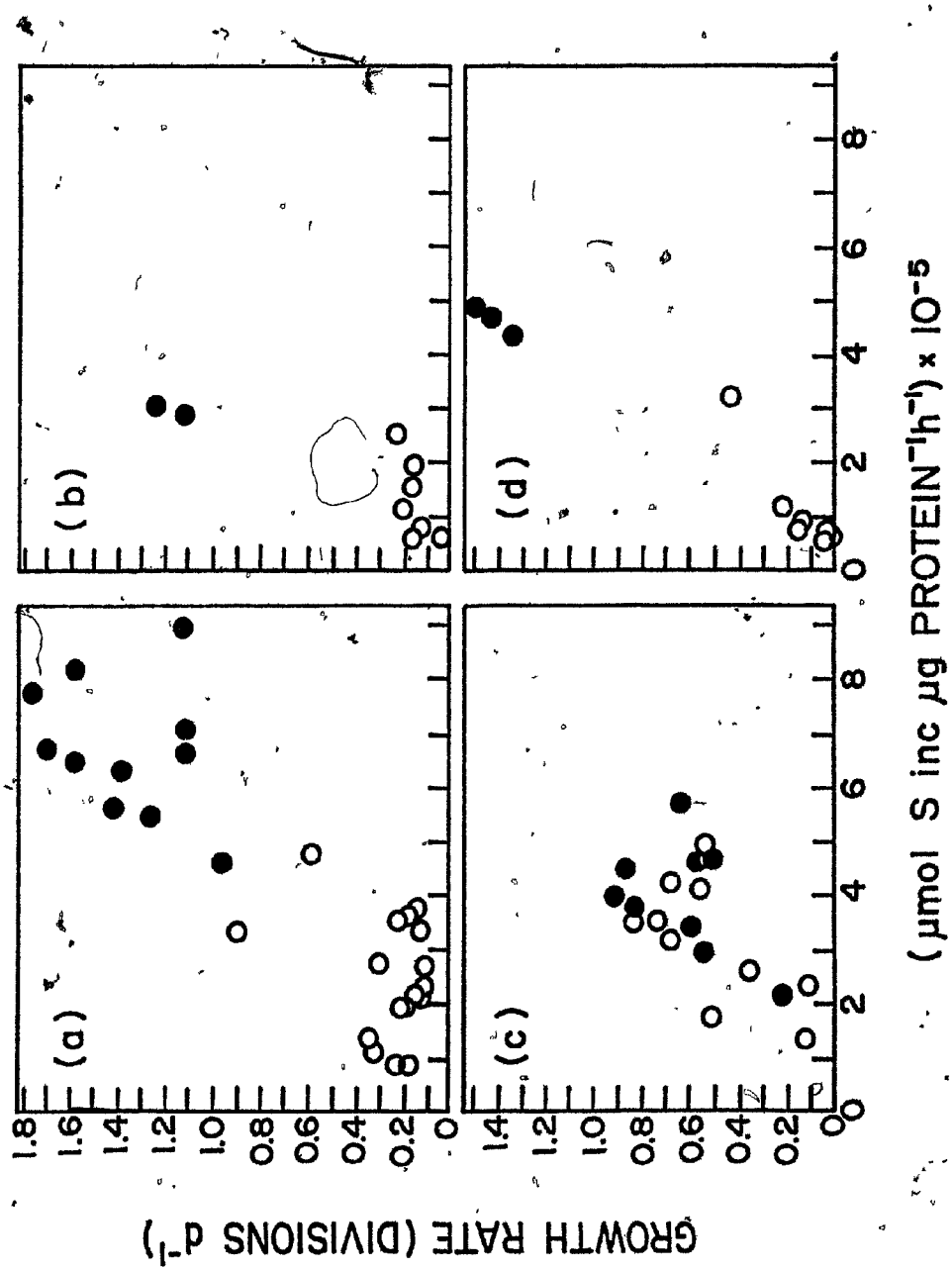

(a)
(b)
(c)
(d)
GROWTH RATE (DIVISIONS d⁻¹)
S inc chl g⁻¹ h⁻¹ (mol/mol)

Figure 26. Regression of division rate of T. weissflogii on ratio of sulfur incorporation:protein (mol/g) for batch culture (a), (b), (d), and chemostat culture experiments (c). Symbols as in Fig. 22.


(a)
(b)
(c)
(d)
GROWTH RATE (DIVISIONS d⁻¹)
(μmol S inc μg PROTEIN⁻¹h⁻¹) x 10⁻⁵

The most useful and practical relationships are between division rate and dark:light ^{35}S incorporation ratio (Fig. 23) or S inc : chl a ratio (Fig. 25). The validity of applying these ratios in the field to determine division rates of natural phytoplankton assemblages would depend on the ability of most species to exhibit similar growth rate to ^{35}S incorporation characteristics.

Specific growth rates in terms of sulfur could be obtained if the sulfur content of particulate material were easily measurable. However, at present there is no rapid, reliable method for determining the sulfur content of such particulate organic matter. One must also be able to distinguish between living and non-living particulate sulfur for accurate determinations of division rate.

Sulfur-35 incorporation as a measure of protein synthesis

Sulfur-35 that is incorporated into the cellular TCA-insoluble fraction would provide a measure of newly synthesized protein. If certain assumptions are made about the sulfur content of bulk protein, and about equilibrium among labelled pools, then rates of ^{35}S incorporation could be converted into rates of protein synthesis.

The bulk protein of phytoplankton contains from 1.7 to 6.6% cysteine plus methionine, by weight (Chau et al., 1967; Ghuecas and Riley, 1969). These amino acids contain one sulfur atom each. I estimate, therefore, that protein contains between 0.9 to 3.3% sulfur, by weight. This

compares with a mean sulfur content of 1.16 ± 0.56 S.D. (n = 26), by weight, for a variety of enzymes derived from animal, yeast and plant cells (Altman and Dittmer, 1964). Assuming that protein contains 2.8% sulfur (protein:sulfur = 36:1, w/w), the rates of sulfur incorporation I found were converted to rates of protein synthesis. Calculated rates of protein synthesis agree reasonably well with measured rates, and show the same general pattern of change during growth in batch culture (Fig. 27). On day 1, the measured rate is only about 20% of the calculated rate. This is due to a drop in protein content per cell occurring one day after inoculation and has been similarly observed by Myklestad (1974) and Falkowski (1977). During this "lag phase", rapid cell division may be supported by consumption of intra-cellular protein reserves rather than by de novo synthesis (c.f., Falkowski, 1977). The ^{35}S data indicate, however, that new protein was being synthesized. It is not known to what extent this lag period in de novo protein synthesis occurs when natural phytoplankton populations are incubated for a 24 h period.

A linear relationship was found between measured and calculated rates of protein synthesis when data was obtained from cells growing exponentially (Fig. 28). Calculated rates exceed measured rates by up to a factor of two. This suggests that some non-protein ^{35}S is included in the

Figure 27. Comparison between measured (▲) and calculated (●) rates of protein synthesis by T. weissflogii growing in batch culture. Measured rates were obtained from daily accumulations of cellular protein concentration. Calculated rates were obtained by converting daily rates of sulfur incorporation, assuming a protein:sulfur ratio of 36:1 (w/w).

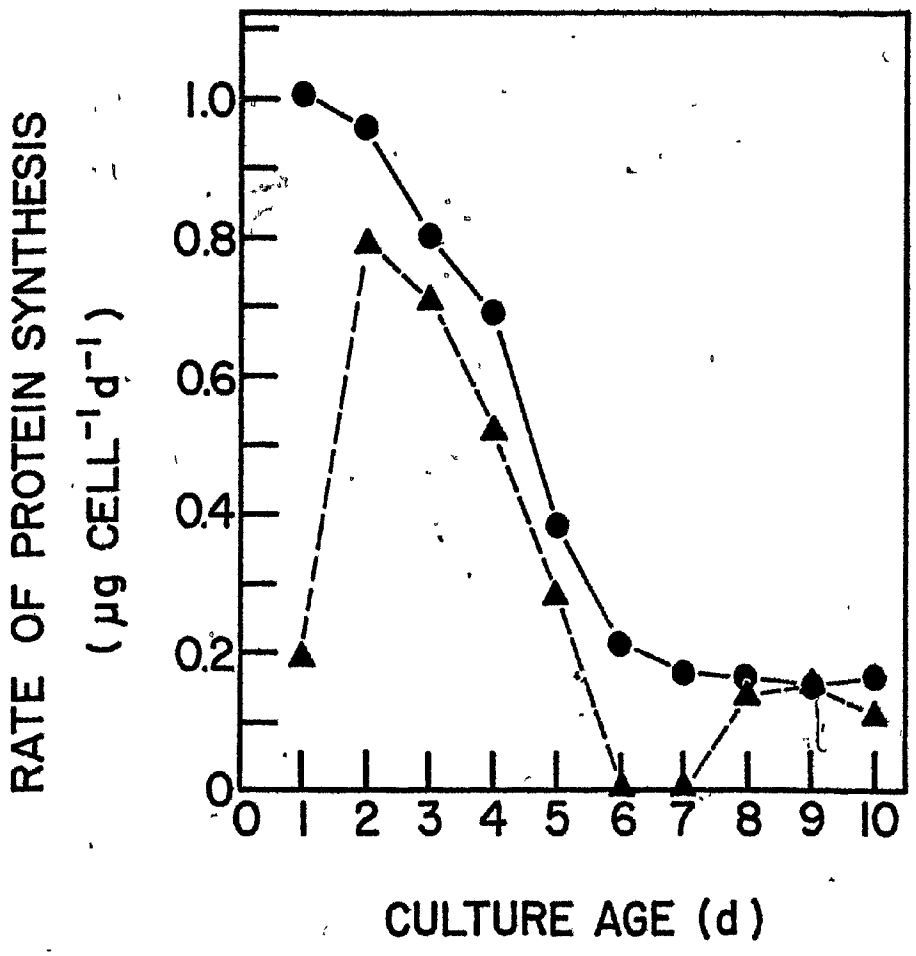
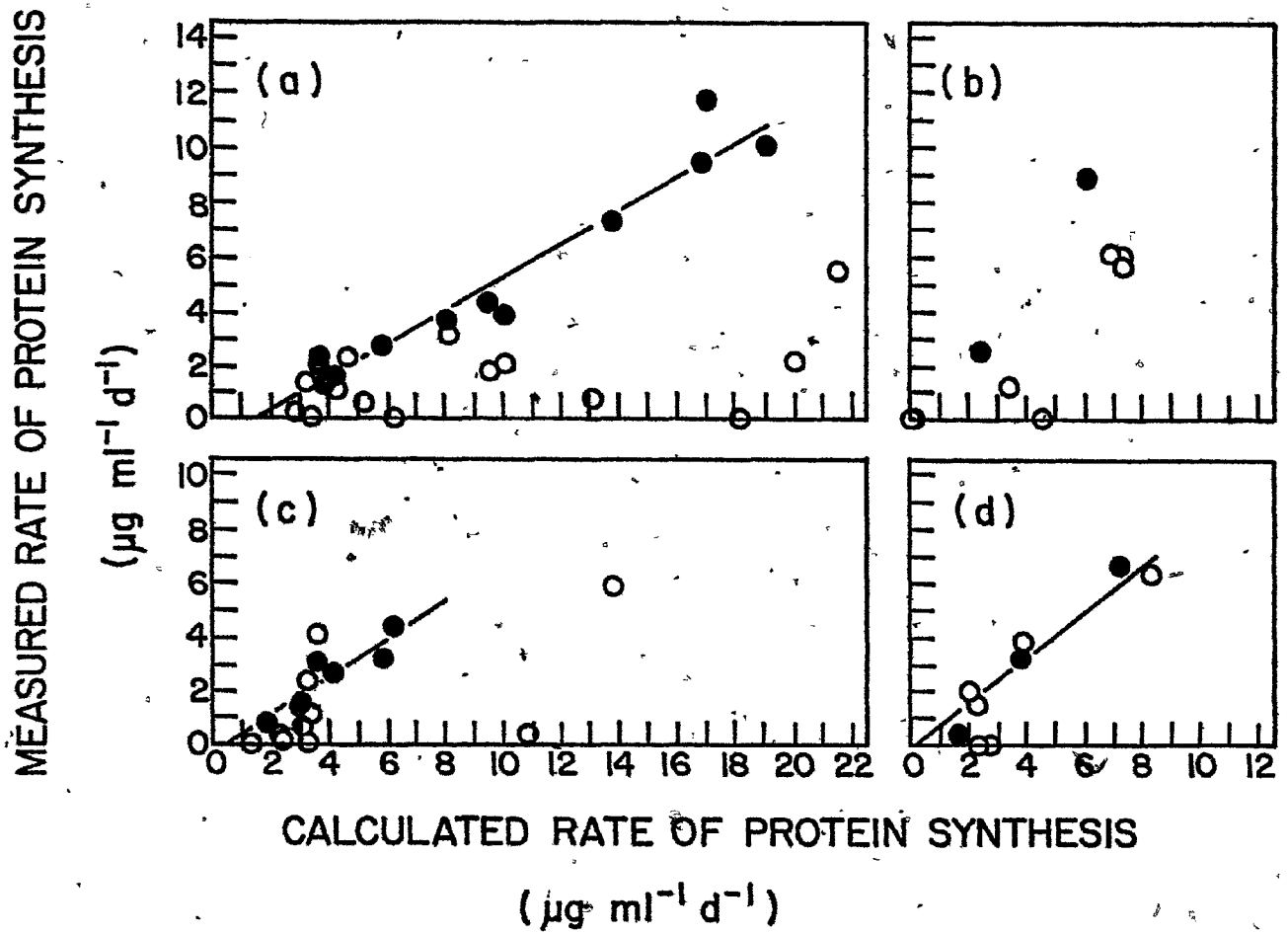

RATE OF PROTEIN SYNTHESIS
(μg CELL⁻¹d⁻¹)
1.0
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10
CULTURE AGE (d)

Figure 28. Regression of measured on calculated rates of protein synthesis by T. weissflogii for batch culture (a), (b), (d), and chemostat culture (c) experiments. Symbols as in Fig. 22. Measured and calculated rates of protein synthesis obtained as in Fig. 27.


MEASURED RATE OF PROTEIN SYNTHESIS
(μg ml⁻¹ d⁻¹)
(a)
(b)
(c)
(d)
CALCULATED RATE OF PROTEIN SYNTHESIS
(μg ml⁻¹ d⁻¹)

protein fraction, as was indicated earlier, or that the protein:sulfur ratio used for the conversion was too high. Using a value of 5.5% for the sulfur content of protein (protein:sulfur = 18:1, w/w) gives a better correspondence between measured and calculated rates.

Improved estimates of the sulfur content of bulk protein in phytoplankton are required to obtain more accurate calculated rates of protein synthesis. The sensitivity of the method would also be greatly improved if the sulfur content of specific proteins was known. For example, the most abundant enzyme in phytoplankton is ribulose bisphosphate carboxylase, accounting for from 30 to 50% of the soluble protein (P. Falkowski, pers. comm.). The cellular concentration of this enzyme may vary according to the physiological status of the cell (Huffaker and Peterson, 1974), accounting in part for the correlation between ^{35}S incorporation and ^{14}C uptake (Fig. 22). One must also consider that proteins of variable sulfur content may be synthesized differentially depending on the cell's physiological state.

The relationship between calculated and measured rates of protein synthesis ceased to be linear during the stationary phase, when calculated rates generally greatly exceeded measured rates (Fig. 28). This suggests that unlabelled protein reserves are degraded faster than new

protein is synthesized. It is likely that this is due to protein turnover, i.e., the renewal of proteins by the process of synthesis and degradation (reviewed by Pine, 1972; Huffaker and Peterson, 1974; Goldberg and Dice, 1974; Davies and Humphrey, 1976). The highest rates of protein degradation might be expected to occur in cells stressed by nitrogen deficiency. Selected enzymes are catabolized for their nitrogen content in favour of synthesis of new enzymes which increase the organism's chance for survival. During active growth, although the protein turnover rate is also believed to be high, the rate of synthesis exceeds that of degradation and protein accumulates.

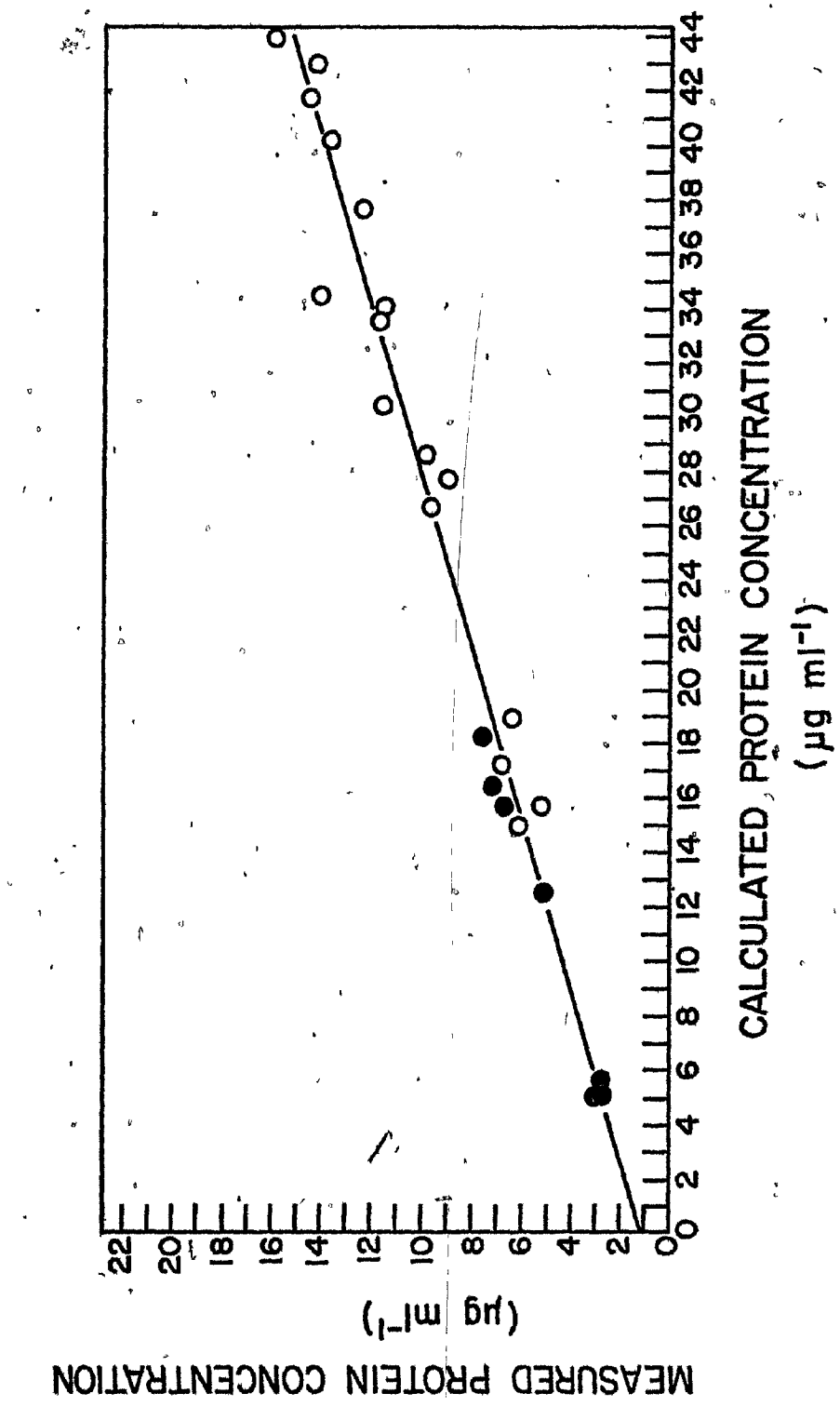
It is difficult to study protein turnover because there is no entirely satisfactory method to circumvent the problem of amino acid recycling (c.f., Davies and Humphrey, 1976). It is generally felt that newly synthesized proteins are as likely to be degraded as old ones, but the issue is far from resolved (Pine, 1972). For these reasons, it is risky to attempt a calculation of the rate of protein turnover from my data. Kemp and Sutton (1971) estimated a 1.1% turnover rate per hour for tobacco plant protein, a value consistent with other studies of higher plants (Oaks and Bidwell, 1970). The protein turnover rate in algae may be much higher because of the organism's requirement to adapt to rapidly

fluctuating environmental factors (c.f., McCarthy and Goldman, 1979; Riper et al., in press).

The longer the incubation period, the greater is the chance for ^{35}S-labelled proteins to be degraded. Long incubations would therefore tend to give a measure of net synthesis of protein (i.e., protein accumulation), while short incubations measure gross protein synthesis (c.f., Strickland, 1960, for studies of photosynthesis). This is illustrated by incubating T. weissflogii for 7 d in radiosulfate. A linear relationship was found between measured and calculated protein concentrations during both exponential and stationary phases of growth (Fig. 29). Because of uncertainty as to which proteins are degraded and when, it is not possible to determine whether a 24 h incubation period provides a measure of net or gross protein synthesis. My data indicates, however, that ^{35}S incorporation gives a better estimation of the rate of protein accumulation during active growth than during senescence. Criteria such as measurement of ratios of dark:light sulfur incorporation, C up : S inc, or S inc : chl a would help to define the phytoplankter's physiological state so that one may decide whether it is appropriate to calculate rates of protein synthesis from ^{35}S data.

Isotopic equilibrium must be reached between the pre-

Figure 29. Regression of measured on calculated protein concentrations of T. weissflogii growing in batch culture in the continuous presence of radiosulfate. Symbols as in Fig. 22. Measured and calculated protein concentrations were obtained from daily accumulations of cellular protein and ^{35}S incorporated, respectively. A protein:sulfur ratio of 36:1 (w/w) was used to convert incorporated sulfur to protein.


MEASURED PROTEIN CONCENTRATION
(μg ml⁻¹)
0 2 4 6 8 10 12 14 16 18 20 22
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
CALCULATED PROTEIN CONCENTRATION
(μg ml⁻¹)

cursor pools and product for the determination of absolute rates of protein synthesis (Oaks and Bidwell, 1970). At equilibrium, the specific activities of the product and precursor should be equal. The time required to achieve equilibrium is a function of the turnover rate of the protein pool. During active growth when the turnover rate is high by virtue of high net rates of protein synthesis, isotopic equilibrium may have been achieved during the 24 h incubation period. However, during the stationary phase when rates of ^{35}S incorporation were low, it is unlikely that sufficient time was available for equilibrium to be reached.

Sulfur-35 incorporation as a field tool

The ^{35}S incorporation technique is designed for ultimate use in the field. Experiments carried out in the North West Arm, in coastal South American waters, and in the Davis Strait demonstrate that ^{35}S uptake and incorporation can be measured in natural marine phytoplankton populations.

Time-series data from the North West Arm show that increases in particulate radioactivity during a 24 h incubation period are reasonable when compared with increases in particulate ^{14}C activity or chlorophyll a concentration (Fig. 20). Molar ratios of C up : S inc (when measured) and S inc : chl a from the three field

areas (Tables 7 - 9) fall within the range of ratios obtained in the laboratory with T. weissflogii (Figs. 24, 25). Division rates calculated from S inc : chl a ratios, however, compare rather poorly with those obtained using ^{14}C uptake and C:chl a ratios (Table 8). Too few data were obtained in the field to make rigorous comparisons of division rates calculated from the dark: light ^{35}S incorporation ratio. However, they appear to be generally lower than division rates calculated by other methods (Table 8). Sulfur-35 uptake data from the Davis Strait provided information about the development of a bloom that could have been obtained otherwise only by sampling over the time-scale of the bloom.

The field results are encouraging considering that data from natural populations of diverse species composition and different geographical locations could be compared at all with data from a laboratory culture of only one diatom, T. weissflogii.

It remains to be determined whether the ^{35}S method for assessing physiological state can be used in the most oligotrophic oceanic areas. Greater than about 10^3 cells must be harvested for an accurate measure of ^{35}S incorporation. Phytoplankton concentrations in the north central Sargasso Sea range from 10^3 to 10^5 cells ℓ^{-1} (Riley, 1957).

Bacterial uptake of radiosulfate may overestimate the measure of ^{35}S by natural assemblages of phytoplankton. This may be particularly important when division rate or physiological state is assessed by measuring ^{35}S incorporation in the dark. Bacterial biomass is 15-35% of the total microbial carbon in coastal waters (Ferguson and Rublee, 1976) but probably less in the open ocean. The metabolic activity of bacteria in these areas is less well known. Experiments in the North West Arm showed that particulate ^{35}S activity and chlorophyll *a* concentration increased at the same rate over a 24 h incubation period (Fig. 20d), suggesting that phytoplankton were primarily responsible for the measured activity.

Physical or chemical methods may be used to alleviate the interference due to bacteria. In this study 1.0 µm Nuclepore filters were used as a compromise for permitting the maximum number of bacteria to pass through the filter while retaining the maximum number of phytoplankton (c.f., Salonen, 1974). Azam and Hodson (1977) found that 10-15% of particulate chlorophyll *a* (presumably microflagellates) passed 1.0 µm Nuclepore filters in a coastal area and 20-40% in offshore waters. About 90% of the heterotrophic activity was by organisms (presumably bacteria) smaller than 1 µm.

Gentamycin, an inhibitor of bacterial protein synthesis (Caskey, 1973) may be used in conjunction with 1.0 µm pore size filters. Laboratory results with gentamycin were unfortunately inconclusive. Although gentamycin at 60 µg ml^{-1} inhibited growth and ^{35}S uptake by bacteria alone (Fig. 18), it failed to do so in the presence of T. weissflogii (Fig. 19). The phytoplankton cells may have metabolized the gentamycin or otherwise reduced its concentration to a level that was no longer inhibitory (c.f., Fig. 18a). There is also an apparent concentration dependency of gentamycin on influencing cellular metabolism of phytoplankton. Berman (1975) found that gentamycin at 20-40 µg ml^{-1} had little effect on photosynthesis by phytoplankton other than blue-green algae. However, I found a stimulation of photosynthetic ^{14}C fixation by phytoplankton from Peruvian waters with 120 µg ml^{-1} of gentamycin (Bedford Institute of Oceanography Data Report, in prepartion). Gentamycin also stimulated ^{35}S uptake and ^{35}S incorporation by T. weissflogii when supplied at greater than 60 µg ml^{-1} (Fig. 19a, Table 6).

Gentamycin and cycloheximide are specific inhibitors of ^{35}S incorporation by bacteria and phytoplankton, respectively (Fig. 18, Table 4). This suggests that the inhibitors, singly and in combination, could complement

the size-fractionation method used to differentiate between bacterial and phytoplankton activity in the sea (c.f., Berman, 1975; Azam and Hodson, 1977).

Finally, the presence of organic sources of sulfur in seawater may lead to an underestimation of ^{35}S incorporation if these non-radioactive sources are preferentially taken up over radiosulfate (c.f., Roberts et al., 1955; Monheimer, 1974b; Jassby, 1975). However, it is unlikely that organic forms of sulfur (e.g., cysteine, methionine, glutathione) are available for uptake by phytoplankton in the sea. Bacteria would probably out-compete the phytoplankton for these substrates at natural concentrations. Most of the remaining sulfur containing organic compounds are readily photo-oxidized (R. Zika, pers. comm.).

Suggestions for further research

Nitrate was the nutrient of interest in this study because of the generally held belief that nitrogen is frequently the important growth-limiting nutrient in the ocean, and because of the requirement for nitrogen in protein synthesis. The effects of silicon and phosphorus deficiencies should also be studied.

Diatoms play a dominant role in the most productive areas of the ocean and have an absolute requirement for silicon (Werner, 1977). Silicon not only is a major

component of diatom cell walls, but also directly or indirectly influences specific metabolic processes. With the onset of silicon deficiency cell division stops, and the net synthesis of protein, among other compounds, is inhibited (Werner, 1977). Decreased rates of ^{35}S incorporation should therefore accompany silicon limitation in diatoms. The effect of silicon deficiency on ^{35}S incorporation by other phytoplankton classes is less clear. If silicon is generally required for protein synthesis, then this should be reflected in rates of ^{35}S incorporation by silicate-deficient cultures. This information is relevant if an assemblage is composed mainly of phytoplankters other than diatoms.

Phosphate is not the major limiting nutrient in the ocean, except perhaps in specialized areas (Perry, 1976; Steemann Nielsen, 1978). It would nevertheless be important to be able to identify phosphate limitation in the field and to understand the effects of phosphate limitation on ^{35}S incorporation.

Phosphate limitation will eventually deplete essential phosphorus reserves (Fuhs, 1969), stopping protein synthesis and cell division. The rate of carbon fixation declines with phosphate limitation, but does not stop. Thus cellular carbon and cell size increase in the absence of cell division (Lehman, 1976). Kylin (1964a; 1964b)

found that phosphate limitation in the freshwater Scenedesmus sp. resulted in decreased protein-S levels but increased amounts of cellular sulfate, soluble reduced sulfur and lipid-S. The ratio of S up : S inc should therefore be greater for phosphate-limited than non-limited cells. Kylin (1964a) also found that sulfate uptake was inhibited by addition of phosphate to phosphorus-limited cultures, due perhaps to a common transporter of these anions (c.f., Hampp and Ziegler, 1977; Lawry and Jensen, 1979). This may provide a test for phosphate limitation in the sea.

The effects of temperature, and light quantity and quality on ^{35}S incorporation should be studied because of their known influence on rates of protein synthesis (c.f., Morris et al., 1974; Morris and Skea, 1978). A more complete understanding of rates of protein turnover at different physiological states, and analytical determinations of the sulfur content of bulk protein and individual major enzymes in phytoplankton would result in better estimates of rates of protein synthesis from ^{35}S data.

Finally, it would be of interest to investigate the kinetics of sulfate uptake which, from the few published reports, appears to conform to Michaelis-Menten type saturation kinetics. The only known study of a marine

phytoplankter is by Deane and O'Brien (1975) who reported a V_{max} of 7.9×10^{-4} pmol SO_4^{2-} cell^{-1} h^{-1} (= 2.4×10^{-3} pmol sulfur cell^{-1} h^{-1}) and a K_m of 32 µM SO_4^{2-} for Pavlova lutheri. The maximum sulfate concentration used in their study was 0.5 mM and it is not clear from their data if this level was saturating, especially when compared to a sulfate concentration of 25 to 30 mM in seawater. Utkilen et al. (1976) found a V_{max} of 0.42×10^{-4} pmol SO_4^{2-} cell^{-1} h^{-1} (=0.13×10^{-3} pmol sulfur cell^{-1} h^{-1}) and a K_m of 0.75 µM SO_4^{2-} for fresh water cyanophyte, Anacystic nidulans. Coughlan (1977) reported a monophasic response for the macroalga Fucus serratus over the range 0.001 to 1 mM sulfate with a V_{max} of 25 µmol SO_4^{2-} g^{-1} fr. wt. h^{-1} and a K_m of 59 µM. I found the velocity of sulfate uptake (equivalent to V_{max} at ambient sulfate concentrations in seawater) by T. weissflogii to range from about 1×10^{-3} to 30×10^{-3} pmol sulfur cell^{-1} h^{-1}, depending on the cell's physiological state.

Many basic questions in phytoplankton ecology require more complete answers. The ^{35}S incorporation technique, if proven satisfactory by additional thorough testing, may be useful in providing some of the answers.

Researchers are currently questioning the degree of limitation of phytoplankton growth by nutrients in oli-

gotrophic areas of the ocean. McCarthy and Goldman (1979) present evidence that phytoplankton cells with a history of nutrient deprivation have an enhanced capacity to take up nitrogenous nutrients that are present only intermittently. They argue that these nutrients could be derived from bacterial remineralization or zooplankton excretion, and would occur on spatial and temporal scales relevant to the phytoplankter but difficult to detect by present-day analytical methods. This short-term enhanced nutrient uptake would probably also go undetected by current techniques which use long incubation times.

Steemann Nielsen (1978) found the growth rate of the freshwater Selenastrum capricornum in dilute batch culture with nitrate and ammonium at the limits of detection, to be about 75% of the optimum. Generation times of several hours were reported for microplankton, presumably phytoplankton, of the Sargasso Sea (Sutcliffe et al., 1970; Sheldon et al., 1973; Sheldon and Sutcliffe, 1978). This time is an order of magnitude less than suggested by other studies in oligotrophic areas (Eppley et al., 1973). These discrepancies may result in part from the use of nutrient or ^{14}C uptake methods to determine growth in previous studies, while Sheldon and Sutcliffe measured increases in POC or ATP. In oligotrophic areas where steady-state conditions for phytoplankton growth may not be established (McCarthy and Goldman, 1979), nutrient uptake as well as

photosynthesis may be uncoupled from growth over time scales less than the generation time of the phytoplankter. Measurement of nutrient uptake or photosynthetic rates obtained after an incubation time of several hours to a day would give a different impression of division rate and physiological state than would a direct measure of increases in biomass. In these situations ^{35}S incorporation, which is more directly related to cell division, may provide a more realistic representation of the events.

If phytoplankton are maintained in a state of at least borderline nutrient sufficiency in the ocean (c.f., Steemann Nielsen, 1978; McCarthy and Goldman, 1979; Goldman et al., 1979), it may be questioned whether phytoplankton exist in a senescent condition. In coastal areas, at least, it appears that phytoplankton pass through exponential, stationary and senescent phases analogous to those in a batch culture (Platt and Subba Rao, 1970; Fogg, 1975; Yentsch et al., 1977). Phytoplankton blooms in coastal waters may sink to the benthos rather than be grazed (Riley, 1956; Platt and Subba Rao, 1970; Walsh et al., 1978; B. Hargraves, pers. comm.). In the open ocean phytoplankton growth and zooplankton grazing are more tightly coupled, and low phytoplankton biomass does not necessarily imply poor physiological condition. When a chlorophyll maximum layer is found, it is not completely understood how the layer is maintained or what the

physiological state of the phytoplankton in the layer is, although based on photosynthetic ^{14}C fixation, it is felt that the population is actively growing (e.g., Eppley et al., 1973). Measurement of ^{35}S incorporation may provide information about these problems that cannot be obtained by existing approaches.

CONCLUSIONS

The ^{35}S incorporation method presented here comes as close as any other method to being able to determine the physiological state and growth rate of marine phytoplankton. An ideal method remains to be found, but were it to exist it should 1) be easy to use, inexpensive, rapid and sensitive, 2) be free of interference by detritus, bacteria and zooplankton, 4) measure parameters that can be correlated with growth rate, 5) provide an instantaneous measurement without containing the sample during an incubation period, 6) be free of species or size-specific growth responses unless that is the aim of the method, 7) be able to assess either overall growth potential or particular growth-determining factors, and 8) provide information on the growth history and predict the direction of change of a phytoplankton population.

Major advantages of the ^{35}S incorporation technique include the specificity of the information and relative ease with which it can be obtained, the lack of perturbation caused by the addition of radiosulfate to the system, the lack of interference by detritus or zooplankton, and the alternative approach it provides to existing methods that seek similar information. On the other hand, the ^{35}S incorporation measured is low as a result of the small quantity of sulfur in protein and the

high ambient sulfate concentration. It is also not yet known how many phytoplankton species share the same or similar relationships among ^{35}S incorporation in the light and dark, ^{35}S uptake, ^{14}C uptake, chlorophyll a, and division rate. This study demonstrated and T. weissflogii, A. carteri, D. tertiolecta and P. lutheri, species representing the four major classes of phytoplankton, had similar patterns and rates of ^{35}S uptake and incorporation during growth in batch culture. Except for P. lutheri, which exhibited a S inc: S up ratio of 0.16 on the basis of only two determinations, the other species showed rates of ^{35}S uptake which were usually about twice the rate of ^{35}S incorporation. It may therefore be easier and more expedient to measure ^{35}S uptake rather than ^{35}S incorporation.

It may be asked what new information is provided by the ^{35}S incorporation technique that cannot be obtained by existing established methods. The technique yields information similar to that obtained by measuring chemical composition (e.g., Sakshaug and Holm-Hansen, 1977), but without interference by detritus or zooplankton. Measurement of photosynthetic carboxylating enzymes in phytoplankton may be a useful indicator of physiological state in natural populations were it not for possible species-specific differences (Glover and Morris, 1979).

The ^{15}N method quantifies the ability of phytoplankton to utilize nitrogeneous nutrients (e.g., McCarthy and Eppley 1972), but is likely to stimulate nitrogen uptake in the process. Measurement of ^{35}S incorporation, on the other hand, indicates the phytoplankter's ability to grow under ambient nutrient conditions. The measurement of photosynthetic ^{14}C fixation was a better indicator of physiological state than had been anticipated. This is by virtue of the decline in photosynthetic rate exhibited during growth in batch culture, also observed by Griffiths (1973) and Morris and Glover (1974). Nevertheless, the rate of ^{35}S incorporation generally declined more rapidly than the rate of ^{14}C uptake. Under conditions of nutrient limitation, therefore, ^{35}S incorporation may give a more realistic measure of phytoplankton growth than would ^{14}C uptake. Sulfur-35 incorporation is a more sensitive indicator of physiological state than is ^{14}C uptake because it may detect the transition between the exponential and stationary growth phases earlier than is possible with ^{14}C uptake. Sulfur-35 is a relatively specific marker for protein, so it is more appropriate to calculate rates of protein synthesis from ^{35}S incorporation than from ^{14}C uptake.

The value of the ^{35}S incorporation technique lies in the alternative approach it provides for determining physiological state, division rate and rate of protein

synthesis by phytoplankton. In this respect, ratios of dark:light ^{35}S incorporation, S inc : chl *a* and perhaps to a lesser degree, C up : S inc, may prove to be useful.

More research is required before the ^{35}S incorporation technique can be recommended for general use in field situations. Aside from rigorous field testing, especially in oligotrophic waters, additional basic physiological research must be carried out. The method for distinguishing between phytoplankton and bacterial growth must be refined. Additional phytoplankton species, including those belonging to different size-classes, should be tested under a variety of environmental conditions to determine the range of the technique's applicability.

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APPENDIX A

Attempts to Eliminate the Retention of Radioactivity by Filters

INTRODUCTION

The measurement of ^{35}S incorporation by marine phytoplankton requires that the ^{35}S-labelled cells be separated from the ^{35}S-containing seawater in which they were incubated. This was accomplished by vacuum filtration. However, during the early stages of the research difficulties were encountered due to unacceptably high filter blanks. Potential sources of errors have previously been reported due to the retention of ^{14}C (Morris et al., 1971a; Nalewajko and Lean, 1972; Williams et al., 1972; McMahon, 1973) and ^{35}S (Jassby, 1975; Jordan et al., 1978; Campbell and Baker, 1978a) onto filters.

This appendix documents the retention of radioactivity onto filters while working with solutions of radiosulfate, and discusses the methods used to deal with the problem. It is hoped that this information will help others to avoid many of the same difficulties.

I wish to thank the members of my committee for suggesting many of the ideas tested herein.

MATERIALS AND METHODS

Radiosulfate (^{35}S as $H_2{}^{35}SO_4$) was obtained from New England Nuclear Corp. (Boston, Massachusetts) in 14 separate batches over a 2-year period. It was necessary to order stock solutions of radiosulfate frequently due to the relatively short half-life ($t_{\frac{1}{2}}$ = 87.2 d) of ^{35}S. Each batch was diluted and treated according to Table A1. The filter retention properties of an ^{35}S batch were tested by filtering a given volume of an ^{35}S plus filtered seawater mixture through a 24 mm diameter filter. The filter was then washed as stated in each experiment, and counted in a scintillation spectrometer as described in the body of this thesis. The radioactivity in the seawater, volume filtered, filter type, pretreatment of the ^{35}S stock solution and filter, and nature of the wash are given in Table A2a-i.

The following filters were tested: glass fiber (Type A-E, Gelman Instrument Co.), polycarbonate membrane (0.4 μm, 1.0 μm, Nuclepore Corp.), cellulose acetate membrane (0.22 μm, 1.2 μm, Millipore Corp.), and silver filters (Selas Flotronics). The dialysis tubing was 1 cm width, 12,000 molecular weight cutoff (Fisher Scientific Co.).

Table A1. Treatment of the $^{35}SO_4^{2-}$ stock solutions received from New England Nuclear Corp.

Batch #	Assay date (d-mo-y)	Quantity Ordered ($mCi\ ml^{-1}$)	Final Activity ($\mu Ci\ ml^{-1}$)	Volume diluted into (ml)	#times filtered (0.22 µm Millipore)	NaCl added (5%)	Dialyzed stock solution	Autoclaved in ampules
1	3-02-76	2	100	19	0	no	no	no
2	5-03-76	10	100	99	8	no	no	no
3	14-06-76	10	200	49	3	no	no	no
4	6-07-76	10	200	49	6	yes	no	no
5	9-08-76	10	200	49	6	yes	no	no
6	6-12-76	10	200	49	6	no	no	no
7	31-01-77	10	200	49	3	no	no	no
8	1-04-77	10	200	50	0	no	yes	yes
9	24-06-77	10	200	50	0	no	yes	yes
10	8-07-77	25	200	125	0	no	yes	yes
11	1-08-77	25	200	125	0	no	yes	yes
12	30-08-77	25	200	125	0	no	yes	yes
13	19-09-77	800	600	1330	0	no	yes	yes
14	3-03-78	225	2250	100	0	no	yes	yes

RESULTS

Filter type

Millipore membrane filters (1.2 μm) retained the most radioactivity (Table A2a: 18-2-76) followed by silver filters (Table A2e: 4-7-77), glass fiber filters (Table A2a: 18-2-76) and Nuclepore filters (Table A2b: 28-5-76). Activity was retained by Celite (diatomaceous earth) as well as by filters (Table A2h: 24-9-77).

Pretreatment of the ^{35}S stock solution

Prefiltration. Progressively less activity was retained by a series of 0.22 μm Millipore filters after sequentially vacuum-filtering the ^{35}S stock solution (Table A3). Filtering the solution more than three times sometimes resulted in an increase in activity retained by the filter.

Starting with experiment 21-6-77 (Table A2c), the radiosulfate stock solution was pressure-filtered immediately prior to use by passage through a 13 mm diameter 0.22 μm Millipore filter mounted on a Swinnex filter holder. Pressure filtration may eliminate particle formation which is otherwise possible when the solution is vacuum-filtered. Pressure filtration resulted in decreased filter blanks (Table A2a: 19-5-76 compared to Table A2b: 20-5-76; Table A2c: 21-6-77; Table A2d: 25-6-77 compared to 26-6-77).

Table A2. Type of filter, pretreatment of the filter, volume and radioactivity of the solution filtered, and type of wash used to obtain the stated radioactivity retained by the filter. N = Nuclepore filter; GF = glass fiber filter; M = Millipore filter; S = silver filter; fsw = filtered seawater; P = prefiltered radiosulfate stock solution; D = dialyzed radiosulfate stock solution; TF = top filter; BF = bottom filter.

Table A2a

Batch #	Date (d-mo-y)	^{35}S activity ($\mu Ci\ ml^{-1}$)	Volume filtered (ml)	Dilute with x ml fsw	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	ml	Filter edge wash solution	ml	Comments	Radio-activity on filter (d.p.m.)
1	18-02-76	0.11	150	0	GF	no	fsw	150	--	0		4919
								300				4750
								450				4521
								600				4824
								750				4432
								900				4596
1	18-02-76	0.11	150	0	M	no	fsw	150	--	0		5422
								300				5200
								450				5798
								600				7506
								750				5019
								900				5486
2	28-05-76	0.01	0.1	0	GF	no	none	0	--	0		216
							fsw	100	--	0		16
							"	"	fsw	20		17
							H_2SO_4	100	H_2SO_4	20		26
							EDTA	100	fsw	20		5
							DDW	100	--	0		435
2	19-06-76	0.10	150	0	GF	no	fsw	200	--	0		894
							"	200	fsw	20		494
							"	400	"	"		505
							DDW	100	"	"		706
							EDTA	100	"	"		423

Table A2b

Batch #	Date (d-mo-y)	^{35}S activity (μCi ml^{-1})	Volume filtered (ml)	Dilute with X ml fsw	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	Filter wash ml	Filter edge wash solution	Filter edge wash ml	Comments	Radio-activity on filter (d.p.m.)
2	20-05-76	0.06	150	0	GF	P	fsw EDTA Na_2SO_4	100 " "	fsw EDTA Na_2SO_4	20 " "		359 380 352
2	28-05-76	3.02	0.3	100	GF	P	fsw	100	fsw	20	Dessicoat GF	405
			1.0	0	N	"	"	"	"	"	" "	53
4	9-07-76	0.75	150	0	N	P	-- fsw "	0 150 150	-- -- fsw	0 0 10		5832 266 12
5	7-10-76	2.23	2.0	100	GF	P	--	0 10 50 100 200 500	--	0		21569 1440 1185 785 621 526
5	7-10-76	2.23	2.0	100	N	P	fsw	0 10 50 100 200 500	--	0		868 233 166 199 134 136
5	8-10-76	1.12	2.0	100	N	P	fsw	100	fsw	0 5 10 15 20 30		361 142 79 73 72 74

Table A2c

Batch #	Date (d-mo-y)	^{35}S activity ($\mu Ci\ ml^{-1}$)	Volume filtered (ml)	Dilute with X ml	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	ml	Filter edge wash solution	ml	Comments	Radio-activity on filter (d.p.m.)
7	29-03-77	2.26	1.0	100	N	P	fsw	100	fsw	15		491
						P/D	"	"	"	"		26
8	21-06-77	1.16	1.0	100	N	D only	fsw	100	--	0	TF	34
			"	"							BF	15
			10	"							TF	57
			"	"							BF	18
			20	"							TF	59
			"	"							BF	28
			50	"							TF	2466
			"	"							BF	157
			100	0							TF	770
			"	"							BF	143
			200	"							TF	7132
			"	"							BF	881
8	21-06-77	1.16	1.0	100	N	P/D	fsw	100	--	0	TF	18
			"	"							BF	15
			10	"							TF	25
			"	"							BF	22
			20	"							TF	61
			"	"							BF	37
			50	"							TF	87
			"	"							BF	59
			100	0							TF	179
			"	"							BF	149
			200	"							TF	347
			"	"							BF	367

Table A2d

Batch #	Date (d-mo-y)	^{35}S activity ($\mu Ci\ ml^{-1}$)	Volume filtered (ml)	Dilute with X ml fsw	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	Filter wash ml	Filter edge wash solution	Filter edge wash ml	Comments	Radio-activity on filter (d.p.m.)
9	25-06-77	1.59	1.0	100	N	D only	fsw	100	--	0	TF	285
			"	"							BF	132
			50	"							TF	1907
			"	"							BF	408
			100	0							TF	2781
			"	"							BF	560
			150	"							TF	32922
			"	"							BF	2941
9	26-06-77	1.53	1.0	100	N	P/D	fsw	100	--	0	TF	62
			"	"							BF	7
			50	"							TF	114
			"	"							BF	27
			100	0							TF	1223
			"	"							BF	304
			150	"							TF	2341
			"	"							BF	2038
9	26-06-77	1.50	1.0	100	GF	P/D	fsw	100	--	0		92
			50	"								753
			100	0								13805
			200	"								30562
9	27-06-77	1.68	1.0	100	N	P/D	DDW	100	--	0	Dilutions and washes with DDW only	58
			50	"								1233
			100	0								989
			200	"								6616

Table A2e

Batch #	Date (d-mo-y)	^{35}S activity (μ Ci ml^{-1})	Volume filtered (ml)	Dilute with X ml fsw	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	Filter wash ml	Filter edge wash solution	Filter edge wash ml	Comments	Radio-activity on filter (d.p.m.)
9	30-06-77	1.72	1.0	100	N	P/D	fsw	100	—	0	^{35}S stock TF	203
			"	"							solution BF	30
			50	"							passed TF	
			"	"							through BF	98
			100	0							Dowex-50 TF	75
			"	"							(H^+) column TF	900
			200	"								746
			"	"								2933
												2998
9	30-06-77	1.94	1.0	100	N	P/D	fsw	100	—	0	^{35}S stock TF	45
			"	"							solution BF	13
			50	"							passed TF	2369
			"	"							through BF	826
			100	0							5 GF TF	5678
			"	"							filters BF	4051
			200	"							TF	7055
			"	"							BF	1356
9	4-07-77	1.84	100	0	N	P/D	2 N H_2SO_4	50	—	0		20
			100	"	S		fsw	10				429
			200	"	S		"	"				7369
10	11-07-77	1.00	1.0	100	N	P/D	fsw	100	—	0		14
			50	"			"	"				80
			100	0			"	"				228
			200	"			"	"				1621
			50	"			2 N H_2SO_4	50	—	0		1
			200	"			"	"				2

Table A2f

Batch #	Date (d-mo-y)	^{35}S activity ($\mu Ci\ ml^{-1}$)	Volume filtered (ml)	Dilute with X ml fsw	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	ml	Filter edge wash solution	ml	Comments	Radio-activity on filter (d.p.m.)
10	13-07-77	1.05	1.0 50 100 200	100 0 " "	N	P/D	fsw	100	--	0	The same ^{35}S solution was passed through the filter to achieve the "volume filtered"	27 139 500 610
10	13-07-77	1.05	1.0 50 100 200	100 0 " "	N	P/D	1 N H_2SO_4	50	--	0	wash filter with 50 ml H_2SO_4 after the 100 ml fsw wash	22 1229 5565 9111
10	27-07-77	7.27	0	0	N	D	fsw	1500	--	0	0.1 min soak in ^{35}S 10 " " 30 " " 60 " " 2 h " 5 " " 24 " " 30 " "	1233 1822 611 2197 1935 1561 2696 2430
10	28-07-77	1.08	150	0	N	P/D	fsw	150	--	0	not soaked 2 d soak in Na_2SO_4	695 665
10	29-07-77	1.08	150	0	N	P/D	fsw	150	-- fsw -- fsw --	0 15 0 15 0	1 d soak in H_2SO_4 " " " " " " " HCl " " " " 10 ml NaOH wash	899 1314 10038 2606 3411

Table A2g

Batch #	Date (d-mo-y)	^{35}S activity ($\mu Ci\ ml^{-1}$)	Volume filtered (ml)	Dilute with X ml fsw	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	Filter wash ml	Filter edge wash solution	Filter edge wash ml	Comments	Radioactivity on filter (d.p.m.)
10	01-08-77	0.81	1.0	100	N	P/D	fsw	150	--	0	15 h sdak in DDW	10
			50	0								223
			100	"								661
			150	"								515
			600	"								1752
10	02-08-77	0.58	1.0	100	N	P/D	fsw	150	--	0	15h soak Na_2HPO_4	37
			50	0							" " "	51
			100	"							" " "	307
			150	"							" " "	413
			150	"							15min" $FeCl_3$	242
			150	"							not soaked	265
			150	"							Dessicoat N filter	314
10	03-08-77	0.55	150	0	N	P/D	fsw	150	--	0	Polycarbonate column	672
						"	"	"		"	Control	813
11	12-08-77	1.01	150	0	N	D	fsw	150	--	0	1% DDW prewash	4042
						"	"	"			Control	2126
						P/D	fsw	150	--	0	1% DDW prewash	1541
						"	"	"	--	0	Control	447
11	12-08-77	1.01	150	0	N	P/D	fsw	150	--	0	EDTA prewash	706
						"	"	"		"	EDTA postwash	929

Table A2h

Batch #	Date (d-mo-y)	^{35}S activity ($\mu Ci\ ml^{-1}$)	Volume filtered (ml)	Dilute with X ml	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	ml	Filter edge wash solution	ml	Comments	radio-activity on filter (d.p.m.)
11	18-08-77	1.01	150	0	N	D	fsw	150	--	0		745
				300		"	"	"				139
				0		P/D	"	"				251
				300		"	"	"				74
12	31-08-77	1.39	150	0	N	P/D	fsw	150	--	0		260
				300		"	"	"	fsw	15		9
12	09-09-77	1.33	150	0	N	P/D	fsw	150	fsw	15		444
				50		"	"	"	"	"		168
				300		"	"	"	"	"		40
13	19-09-77	3.72	150	300	N	P/D	fsw	150	fsw	15		328
		1.79		"			"	"	"	"		1445
		"		"			"	"	"	"		4443
13	24-09-77	1.70	150	0	Celite on GF	P/D	fsw	150	--	0	activity on celite	8863
											" " GF	3944
13	07-10-77	1.55	150	0	N	P/D	fsw	150	fsw	15	control	2375
		"	"	"	"	"	"	"	"	"	1st evaporation	442
											2nd "	1031
											3rd "	4704

Table A2i

Batch #	Date (d-mo-y)	^{35}S activity ($\mu Ci\ ml^{-1}$)	Volume filtered (ml)	Dilute with X ml fsw	Filter type	P or D $^{35}SO_4^{2-}$	Filter wash solution	ml	Filter edge wash solution	ml	Comments	radio-activity on filter (d.p.m.)
13	28-11-77	2.00	150	100	N	n.a.	fsw	150	fsw	15	filter holder not washed	542
											filter holder not washed	313
											filter holder not washed	864
											filter holder washed	48
												36
												32
13	12-12-77	2.60	100	100	N	P/D	fsw	100	fsw	15	filter holder washed	23
											filter holder washed	29
											filter holder washed	30
13	27-07-78	1.52	2.0	100	N	P/D	fsw	100	fsw	20	No T. weissflogii	17
											2.4×10^4 cells ml^{-1}	31
											4.8 " " "	22
											9.6 " " "	22
											1.4 " " "	10
											1.9 " " "	27
											2.4 " " "	12
~~14~~	03-04-78	2.19	200	100	N	P/D	fsw	100	fsw	20	filter holder not washed	421
											filter holder not washed	459
												512
											filter holder washed	23
											"	29
												30

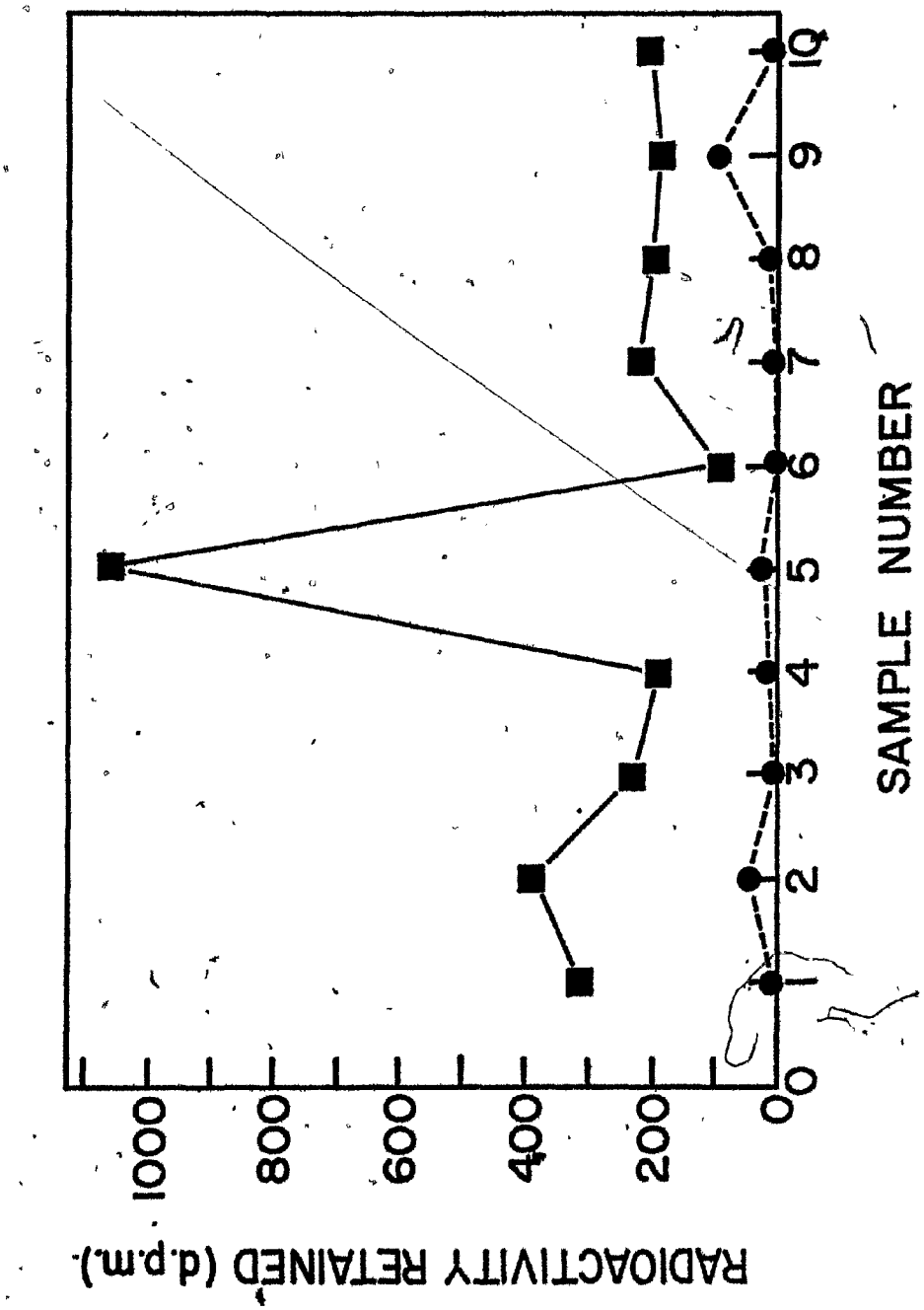
Table A3. Radioactivity retained on a 0.22 μm Millipore filter. The radiosulfate stock solution from batches 1-3, treated as described in Table A1, was sequentially passed through three filters. Each filter was washed with 100 ml of filtered seawater prior to counting. In addition, the filter edges were rinsed with 20 ml of filtered seawater when batch 3 was filtered.

Filter	Radioactivity retained on the filter (d.p.m.) Batch 1	Batch 2	Batch 3
1	2142800	1570000	200800
2	826300	286000	69400
3	84700	86000	17900

Dialysis. Dialysis is an alternative way to eliminate radioactive particulate material from ^{35}S stock solution. The ^{35}S solution received from the manufacturer is dialyzed for about 24 h against a volume of sterile distilled water which gives the desired stock solution activity at equilibrium. Before the stock solution was dialyzed, an order of magnitude more activity was retained on a top filter than on a bottom filter when the solution was passed through two Nuclepore filters (Fig. A1). After dialysis, the activity retained on the top filter was reduced by an order of magnitude (Table A2c: 29-3-77).

Ion exchange. Ion exchange methods were attempted to eliminate possible soluble radioactive contaminants from the ^{35}S stock solution. Passing the stock solution through a Dowex-50 (H^+) column did not reduce the activity retained by the filter (Table A2e: 30-6-77). Polycarbonate Nuclepore and glass fiber filters were efficient adsorbers of activity. The ^{35}S stock solution was therefore passed through these materials in an attempt to strip any radioactive contaminant from the solution. Passing the stock solution through a column containing polycarbonate shavings or through a stack of five glass fiber filters did not reduce the filter blank (Table A2e: 30-6-77; Table A2g: 3-8-77).

Fig. A1. Retention of radioactivity on the top (■) and bottom (●) filter resulting from passing 2.0 ml of radiosulfate (2.3 μC ml^{-1}) plus 100 ml of filtered seawater through two 1.0 μm Nuclepore filters. The filters were washed with 100 ml of filtered seawater, and the filter edges were rinsed with 15 ml of filtered seawater. The radiosulfate stock solution (batch 7) was not dialized or pre-filtered prior to use.


RADIOACTIVITY RETAINED (d.p.m.)
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
9
10
SAMPLE NUMBER

Effect of washing the filter

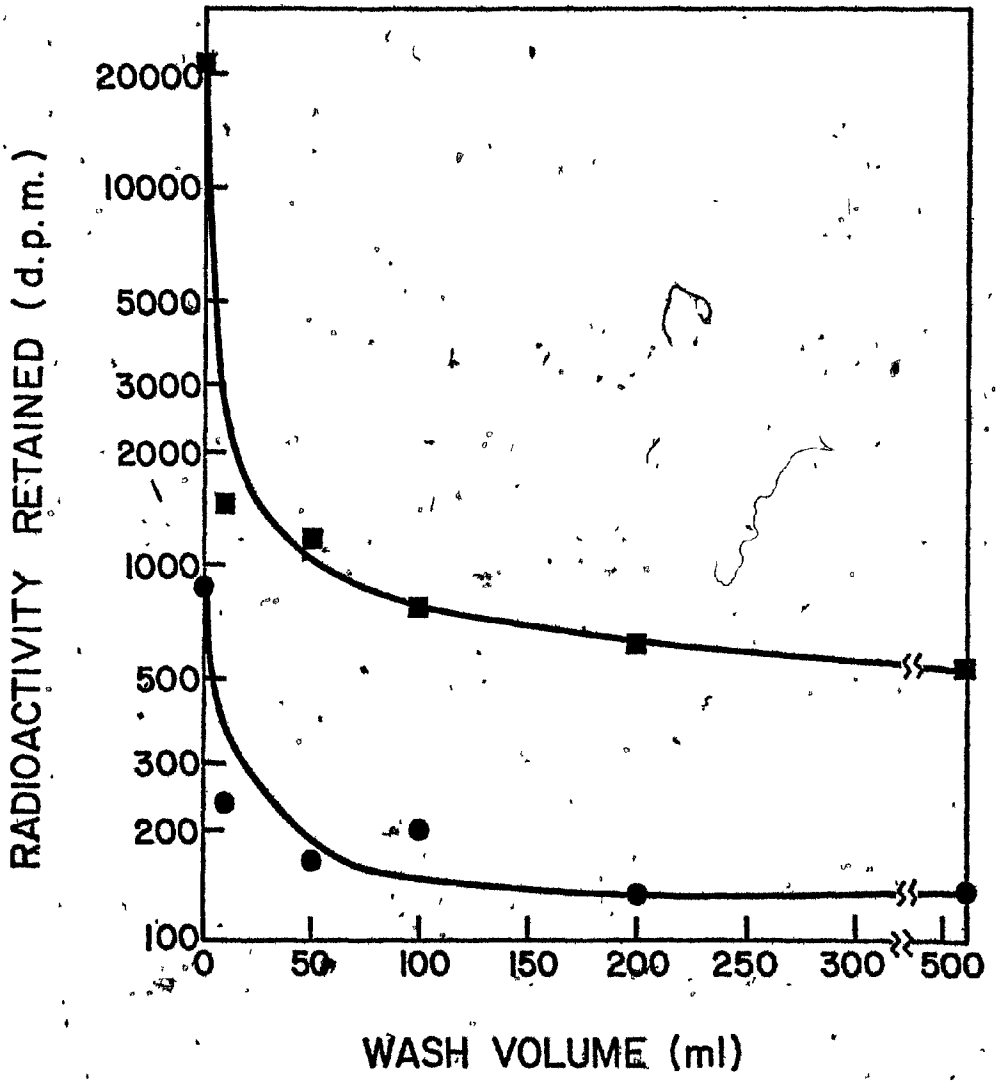
Washing the filter with increasing volumes of filtered seawater greater than 50-100 ml did not further decrease the activity retained by the filter (Fig. A2; Table A2a: 18-2-76; Table A2b: 7-10-76). Up to 2 ℓ of seawater were used as a wash without further success (Table A2g: 2-8-77).

A wash containing Na_2SO_4 (2% w/v, and a saturated solution), Na_2EDTA (0.4%, 1%, and a saturated solution), H_2SO_4 (1N), NaOH (1 N), HNO_3 (conc.), CS_2, benzene or toluene did not markedly decrease the activity retained (Table A2a: 18-5-76; 19-5-76; Table A2b: 20-5-76; Table A2f: 13-7-77; 29-7-77; Table A2g: 12-8-77; Table 2h: 15-8-77). Activity was decreased when a 2 N H_2SO_4 wash was used (Table A2e: 4-7-77; 11-7-77). However, this H_2SO_4 concentration would probably dissolve the labelled phytoplankton cells.

Effect of rinsing the filter edge

Rinsing that part of the filter edge covered by the lip of the filter chimney with filtered seawater from a squeeze bottle had a variable effect on the activity retained, depending on the ^{35}S batch used. Rinsing the filter edges when batches 4 and 5 were used decreased the radioactivity retained (Table A2b: 9-7-76; 8-10-76). A rinse with more than about 15 ml of seawater did not

Fig. A2. Retention of radioactivity on Gelman type A-E glass fiber (■) and 1.0 µm Nuclepore (●) filters as a function of wash volume. The wash solution was filtered seawater. Filter edges were not rinsed. The radiosulfate stock solution (batch 5) was prefiltered and 2.0 ml of a 2.23 µCi ml^{-1} solution of radiosulfate, diluted to 100 ml with filtered seawater, was passed through the filters.


RADIOACTIVITY RETAINED (d.p.m.)
20000
10000
5000
3000
2000
1000
500
300
200
100
0
50
100
150
200
250
300
500
WASH VOLUME (ml)

further decrease the activity (Fig. A3: Table A2b: 8-10-76). No decrease in activity was found with ^{35}S batches 2, 8 and 10 when the filter edges were rinsed (e.g., Table A2c: 29-3-77). Rinsing the filter edges with EDTA or Na_2SO_4 did not decrease the activity (Table A2b: 20-5-76).

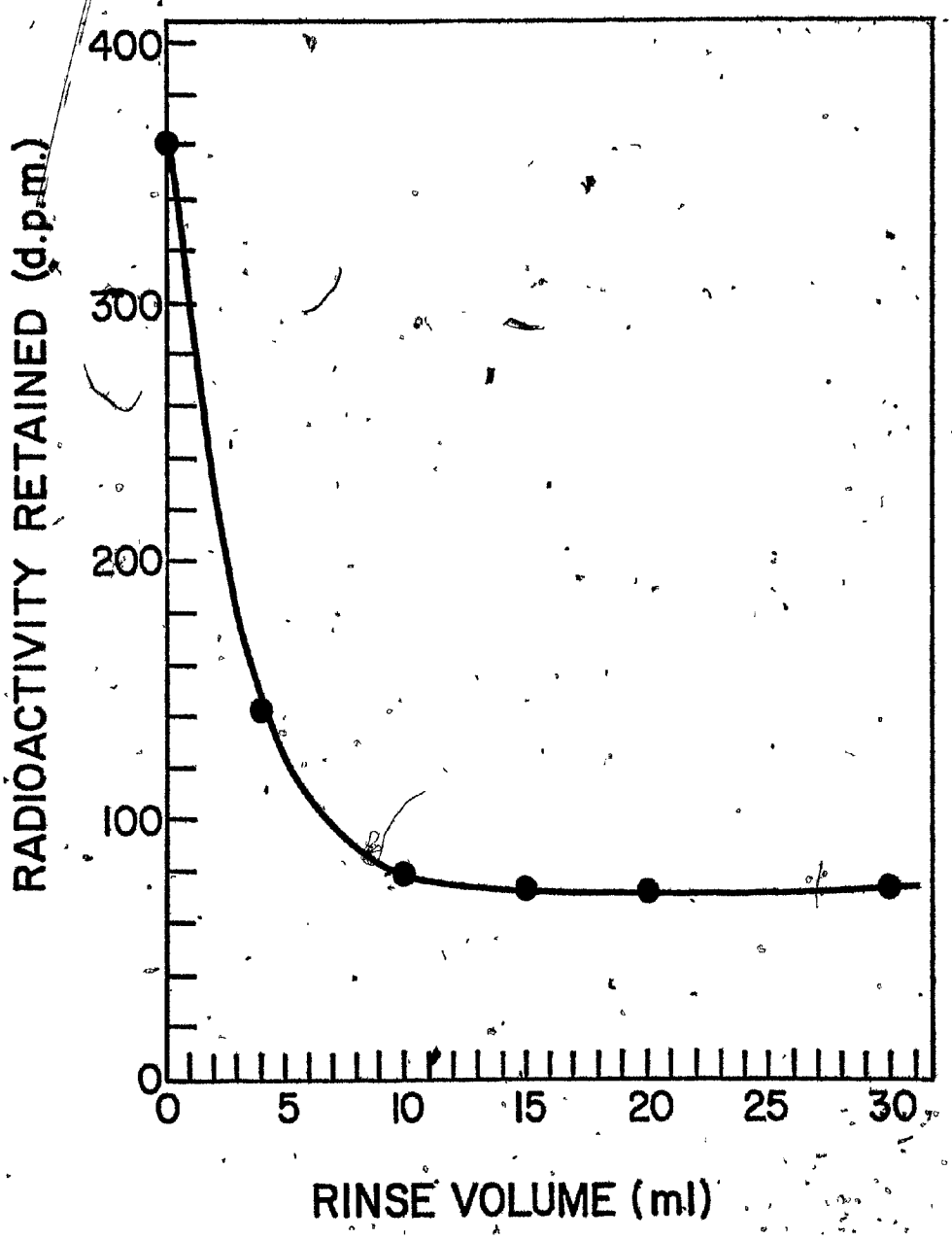
Effect of pretreatment of the filter

It was hypothesized that active sites on the filter could be blocked by pretreatment with various ions. The sites on Nuclepore filters were saturated with radioactivity within the first 0.1 min of contact with the ^{35}S stock solution (Table A2f: 27-7-77). Coating the filter with Dessicoat (Beckman Instrument Co.) prior to filtration did not reduce the activity (Table A2b: 28-5-76); Table A2g: 2-8-77). Soaking the filters in saturated Na_2SO_4, Na_2PO_4, $FeCl_3$, concentrated H_2SO_4, HCl, or sterile filtered seawater, did not reduce the filter blank (Table A2f: 28-7-77; 29-7-77; Table A2g: 1-8-77; 2-8-77). Pretreatment of the filter with saturated EDTA, up to 1 ℓ of filtered seawater or super-Q distilled water increased the filter blank (Table A2g: 1-8-77; 12-8-77).

Effect of filtering increasing volumes of ^{35}S

A positive, linear relationship was found between the volume of radiosulfate plus seawater filtered and activity retained on the Nuclepore filter when the

Fig. A3. Retention of radioactivity on 1.0 μm Nuclepore filters as a function of volume of filtered seawater used to rinse the filter edge. A squeeze bottle delivered about 1 ml of filtered seawater rinse for each turn around the filter edge. The radiosulfate stock solution (batch 5) was prefiltered and 2.0 ml of a 1.12 $\mu Ci \cdot ml^{-1}$ solution of radiosulfate, diluted to 100 ml of filtered seawater, was passed through the filters. Each filter was washed with 100 ml of filtered seawater prior to rinsing the filter.

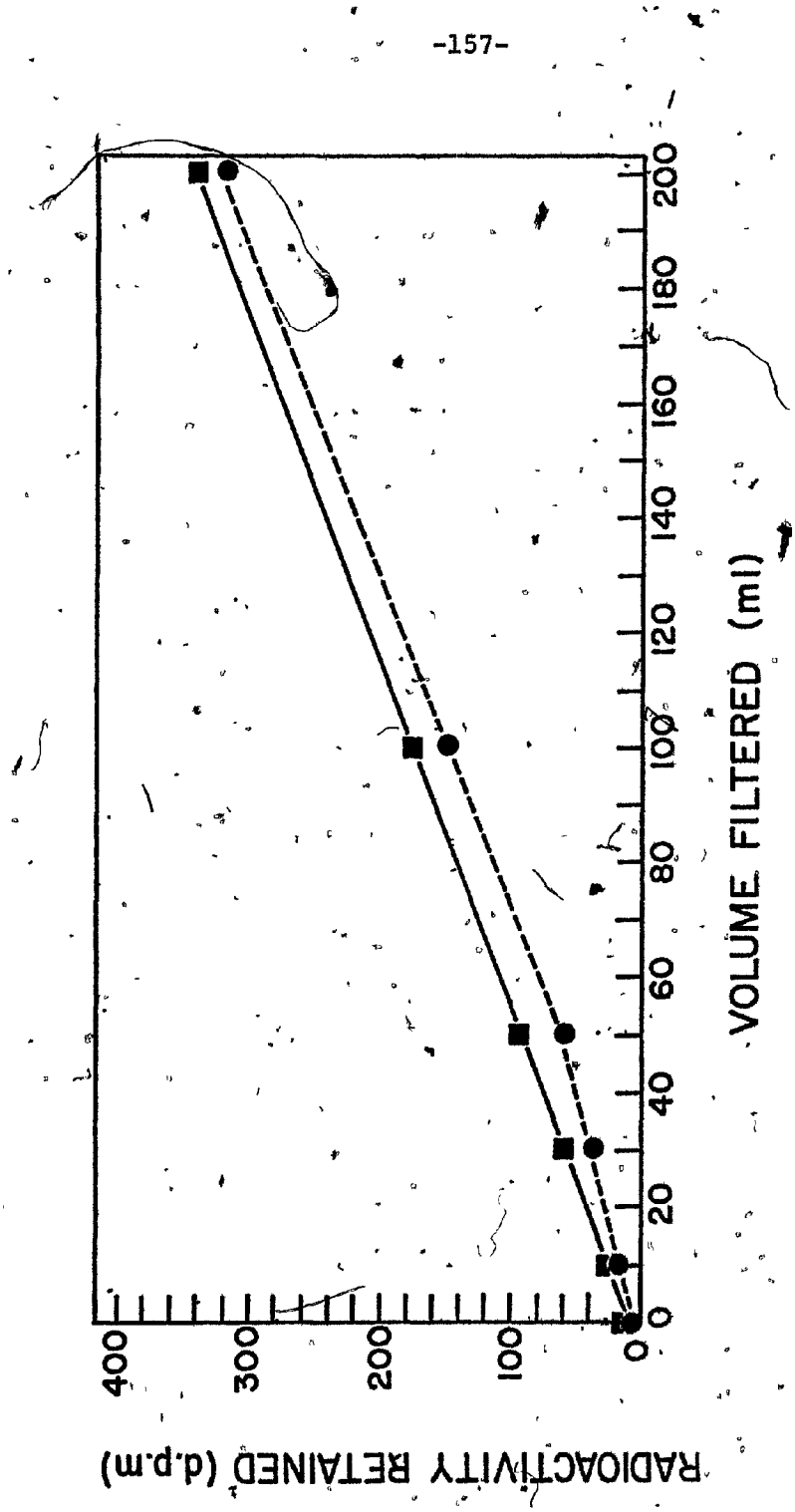

RADIOACTIVITY RETAINED (d.p.m.)
400
300
200
100
0
0
5
10
15
20
25
30
RINSE VOLUME (ml)

filter holder was not washed prior to each filtration (Fig. A4; Table A2c: 21-6-77; Table A2d: 25, 26, 27-6-77; Table A2e: 30-6-77; 11-7-77; Table A2f: 13-7-77). Radioactivity retained on the top and bottom filter was not markedly different when increasing volumes were filtered (Fig. A4). A similar relationship was found when increasing volumes of ^{35}S in distilled water were filtered (Table A2d: 27-6-77), indicating that the retention problem is not a function of interactions among components in seawater. When the same $^{35}SO_4^{2-}$ plus seawater solution was passed several times through the same filter to obtain the given volume of ^{35}S filtered, the filter became saturated with radioactivity after 50-100 ml of solution had passed through (Table A2f: 13-7-77).

Effect of diluting the ^{35}S solution

Diluting the $^{35}SO_4^{2-}$ plus seawater solution with at least 100 ml of filtered seawater prior to filtration resulted in less radioactivity retained than if the solution were added directly to the filter. A 300 ml dilution was more effective in reducing the radioactivity than a 50 ml dilution (Table A2h: 9-9-77). Dilution resulted in lowered filter blanks using batches 11 and 12 (Table A2h: 18-8-77; 31-8-77; 9-9-77), but not batch 13 (Table A2h: 19-9-77).

Fig. A4. Retention of radioactivity on the top (■) and bottom (●) 1.0 μm Nuclepore filter as a function of volume of radiosulfate solution (1.16 μCi ml^{-1}) filtered. The filters were washed with 100 ml of filtered seawater, but the filter edges were not rinsed. The radiosulfate stock solution (batch 8) was dialized and prefiltered prior to use.


RADIOACTIVITY RETAINED (d.p.m)
400
300
200
100
0
0
20
40
60
80
100
120
140
160
180
200
VOLUME FILTERED (ml)

Evaporation of the ^{35}S stock solution

Five ml of ^{35}S stock solution were evaporated to dryness at 65°C for 5 h in an attempt to remove possible volatile radioactive contaminants. The residue was reconstituted with distilled water, added to 150 ml of seawater, and filtered. This evaporation-treated solution showed an order of magnitude decrease in radioactivity retained (Table A2h: 7-10-77), although the radioactivity was still an order of magnitude greater than desired. A second evaporation, and a third evaporation with HCl resulted in an increase in radioactivity retained.

Effect of washing the filter holder

Considerable radioactivity (up to 860 d.p.m.) was retained by a Nuclepore filter when seawater containing no ^{35}S was filtered (Table A2i: 28-11-77). In this case the filter holder (i.e., stainless steel support screen plus polyethylene filter chimney) was not carefully washed after exposure to seawater containing ^{35}S. When the filter holder was washed with either seawater or tap water, the radioactivity retained by the filter was reduced by an order of magnitude to an acceptable level (Table A2i: 28-11-77), even when 100 ml of $^{35}SO_4^{2-}$ plus seawater were filtered (Table A2i: 12-12-77).

These results were repeated using batch 14 (Table A2i: 3-4-78). The amount of radioactivity retained did not vary considerably with increasing concentrations of phytoplankton (Table A2i: 27-7-78).

Differences in the quality of the ^{35}S batches tested

A summary of the differences in quality of each ^{35}S batch is given in Table A4. All of the batches tested gave acceptably low blanks when the stock solution was dialyzed and/or prefiltered provided that about 2 ml or less of $^{35}SO_4^{2-}$ plus seawater were filtered. Batch 4 gave low values without dialysis of the stock solution when 150 ml were filtered. Batches 11 and 12 gave acceptable values when the radioactive solution was diluted to 300 ml prior to filtration. Glass fiber filters retained more radioactivity than Nuclepore filters using batches 1, 2 and 5, but the activity retained by both kinds of filters was the same for batches 4 and 9. Rinsing the filter edges decreased the filter retention in batches 4 and 5, but not in batch 7. Batches 13 and 14 provided low filter blanks when the filter holder was carefully washed prior to use.

DISCUSSION

Several lines of evidence suggest that three factors could contribute to the retention of radio-

Table A4. Summary of the variations in the quality, as indicated by the retention of radioactivity onto filters, of the individual batches of radiosulfate stock solution.

Batch #	Quality of the Batch
1	Poor (~6000 d.p.m.) when stock solution not prefiltered or dialyzed
2	Poor (~600 d.p.m.) when filter 150 ml (GF filter); no prefiltration
	O.K. (~20 d.p.m.) when filter 0.3 ml (Nuclepore); prefiltration
3	O.K. (~20 d.p.m.) when filter 0.1 ml (Nuclepore); prefiltration
4	O.K. (~15 d.p.m.) when filter 150 ml; prefiltration; filter edge rinse
5	O.K. (~70 d.p.m.) when filter 2 ml; prefiltration; filter edge rinse
6	Not tested
7	O.K. (~70 d.p.m.) when filter 2 ml; dialysis; prefiltration. Filter edge rinse no difference
8	O.K. (~50 d.p.m.) when filter 20 ml; dialyis; prefiltration.
9	O.K. (~70 d.p.m.) when filter 1 ml; dialysis; prefiltration. No difference between GF and Nuclepore filters
10	O.K. (14-80 d.p.m.) when filter 50 ml; dialysis; prefiltration
11	Poor (~600 d.p.m.) when filter 150 ml without 300 ml fsw dilution
	O.K. (~70 d.p.m.) when filter 150 ml with 300 ml fsw dilution
12	O.K. (9-40 d.p.m.) when filter 150 ml with 300 ml fsw dilution
13	Poor (300-30000 d.p.m.) when filter 150 ml and treated as above
	O.K. (~30 d.p.m.) if filter holder washed prior to use
14	O.K. (~30 d.p.m.) when filter 200 ml; dialysis; prefiltration; 100 ml fsw dilution

activity by filters: 1) radioactive particulate material, 2) radioactive soluble contaminant(s) in the radiosulfate stock solution, and 3) radioactivity that was improperly rinsed from the filter and/or filter holder. In addition, it appears that the quality of each ^{35}S batch received from the manufacturer may vary.

The preliminary filtering of the radiosulfate stock solution through 0.22 μm Millipore filters showed a decreasing amount of activity retained from the first to the third filter (Table A3), suggesting that radioactive particulates were being removed from the stock solution. This was also reported by Jordan et al. (1978). Dialysis of the stock solution also resulted in a lower filter blank, and prefiltering the dialyzed stock solution prior to use resulted in a further reduction in the blank. The high variability sometimes observed in filter retention values could be caused by trapping the occasional particulate radioactive contaminant. When a radioactive solution was passed through two filters, more radioactivity was retained on the top than on the bottom filter. This could be due to trapping radioactive particulate material or to adsorption of radioactivity on the top filter. That the difference in radioactivity between

the top and bottom filters was minimized by prefiltering the ^{35}S stock solution, indicates that particulate material was removed by prefiltration. Some of the radioactive particulates found in batches 1-7 (Table A1) may have been due to growth of bacteria in the stock solutions, as these solutions were not autoclaved in ampules.

It is not likely that ionic radiosulfate is adsorbed to the filter. A wash containing carrier sulfate should displace sulfate on the filter by ionic exchange. However, up to 2 ℓ of seawater wash, containing about 2.7 g l^{-1} of sulfate, or a wash containing 2% (w/v) Na_2SO_4 did not remove the radioactivity. Saturating active sites on the filter with solutions of Na_2SO_4, H_2SO_4 or seawater also did not prevent radioactivity from being adsorbed. Nevertheless, Nuclepore filters retained less radioactivity than other filters tested. This may be due to the thinness of Nuclepore filters and the lower area available for adsorption. Comparable results were reported by Jordan et al. (1978). They also recommended that Nuclepore filters be dissolved in 1 ml of phenethylamine before addition of the scintillation cocktail to further reduce the background counts by 10-25%.

Added radiosulfate was present in a constant

proportion to carrier sulfate in seawater. Filtering increasing volumes of a seawater solution containing radiosulfate should therefore result in a constant amount of activity retained. This was not the case, as a positive linear relationship was found between the volume filtered and radioactivity retained. Similar results were reported by Campbell and Baker (1978a) for Sartorious filters. This relationship is not possible if the ion being adsorbed onto the filter is radiosulfate, and suggests that another ion not present in seawater is being adsorbed. The possibility was ruled out that radioactive elemental sulfur, which is insoluble in water, was retained by the filter. A wash containing CS_2, benzene or toluene, which solubilize elemental sulfur, did not reduce the radioactivity adsorbed. I would not expect elemental sulfur to be in the stock solution because oxygen present in water is sufficient to keep sulfur oxidized to sulfate (Koski, 1949).

Radiosulfate is manufactured by neutron irradiation of KCl (Herber, 1962). Three acknowledged radioactive byproducts of this procedure are: ^{42}K (γ, $t_{1/2}$ = 12.4h), ^{36}Cl (β^-, $t_{1/2}$ = 4 x 10^5 y, 0.714 Mev) and ^{32}P (β^-, $t_{1/2}$ = 14.3 d, 1.71 MeV). The half-life of ^{42}K is so short that it is undetectable when received from the

manufacturer. The trace amount of ^{32}P (11.5 ppm) is removed by an alumina anion-exchange column by the manufacturer (D. Peterson, New England Nuclear, pers. comm.) and could not be detected with a scintillation counter. Radioactivity due to ^{32}P would diminish in several weeks, but this was not observed. Soaking the filters in a carrier phosphate solution did not prevent adsorption of radioactivity. Some radioactivity was detected in the channel set for ^{36}Cl, but this may be due in part to interference with ^{35}S (0.167 MeV). Elimination of ^{36}Cl by evaporating the ^{35}S stock solution (Herber, 1963) failed to reduce the radioactivity in the ^{36}Cl channel or the filter bank. As the mean filter retention value for each ^{35}S batch varied greatly, the hypothesized radioactive contaminant may be present in variable amounts depending on the batch used. Reuveny and Filner (1976) reported the presence of an unspecified radiochemical impurity in $H_2{}^{35}SO_4$ purchased from New England Nuclear, which was apparently not present in $Na_2{}^{35}SO_4$.

After removal of radioactive particulates by dialysis and prefiltration, the most likely explanation for the high filter blank is the presence of radiosulfate that was not properly rinsed from the area of the filter. Some of the radioactivity is removed by washing the filter

with at least 100 ml of filtered seawater. Another portion is removed by rinsing the edge of the filter hidden by the lip of the filter chimney during the seawater wash. Jordan et al. (1978) and Campbell and Baker (1978a) accomplished the same effect by excising and discarding the outer portion of the filter. The remainder of the radioactivity on the filter and much of the variability was reduced by a careful rinse of the filter holder with tap or seawater between each filtration. This prevented drops of radioactive solution located under the lip or along the inner surface of the filter chimney from coming in contact with the filter. Diluting the $^{35}SO_4^{2-}$ plus seawater solution prior to filtration with 100 ml of filtered seawater while in the filter chimney probably accomplished the same effect, and may therefore not be necessary.

The following procedures are recommended to reduce the amount of radioactivity retained by filters when working with radiosulfate solutions (c.f., Jordan et al., 1978):

1) Immediately upon receipt from the manufacturer, dialyze the $^{35}SO_4^{2-}$ solution against a volume of sterile distilled water that will give the desired stock solution radioactivity at equilibrium, then seal in ampules and autoclave.

2) Pressure-filter the dialyzed $^{35}SO_4^{2-}$ stock solution through a 0.22 µm Millipore filter mounted in a Swinnex filter holder prior to use,

3) Use polycarbonate Nuclepore filters rather than other membrane or glass fiber filters,

4) Rinse the filter holder and forceps with tap or seawater prior to each filtration,

5) Dilute the $^{35}SO_4^{2-}$ plus seawater solution with at least 100 ml of filtered seawater in the filter chimney prior to filtration,

6) After filtration, wash the filter with at least 100 ml of filtered seawater, rinse the inside surface of the filter chimney with filtered seawater, remove the chimney and rinse the outer edge of the filter with at least 20 ml of filtered seawater from a squeeze bottle.

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APPENDIX B

Use of Radiosulfate to Measure Grazing Rates by Temora longicornis, a Marine Copepod

INTRODUCTION

The feeding by zooplankton on phytoplankton can be measured by 1) changes in particle counts, chlorophyll a or carbon, 2) activity of digestive enzymes, or 3) by labelling food itmes with vital stains or radioisotopes. Of the radioisotopes, ^{14}C (e.g., Adams and Steele, 1966; Daro, 1978) and ^{32}P (e.g., Marshall and Orr, 1961; Berner, 1962) are the most commonly used. This appendix documents the use of ^{35}S to measure the filtration and ingestion rates by the copepod Temora longicornis feeding on a laboratory culture of the diatom Thalassiosira weissflogii (= fluviatilis).

The use of radioisotopes to measure grazing rates has been criticized on grounds that the isotope may be rapidly excreted or respired by the zooplankter, thus underestimating assimilation efficiencies (c.f., Conover and Francis, 1973). Sulfur-35 may be more advantageous than ^{32}P or ^{14}C in this respect because it is less rapidly excreted and is not respired. I thank Dr. G. A. Riley for suggesting this experiment.

MATERIALS AND METHODS

Thalassiosira weissflogii was grown for 4 d to the exponential growth phase in f/2 medium containing 1.3 μCi ml^{-1} of radiosulfate. Three ml of labelled culture were diluted to 150 ml with filtered seawater to give an initial cell concentration of 5.2×10^3 cells ml^{-1}. Five adult male Temora longicornis collected in Bedford Basin, Halifax, by Mark Huntley of this laboratory were starved for 24 h then placed in each 150 ml bottle containing labelled culture. After incubation for 24 h in the dark at 10°C on a rotating plankton wheel, the zooplankton were trapped on a 150 μm mesh, rinsed with about 5 ml of filtered seawater and placed in scintillation fluor for determination of radioactivity (see thesis text). The ^{35}S activity of the food organism was determined by filtering 2 ml of fecal pellet-free culture as described in the body of this thesis. Cell numbers were measured with a Model Z_B Coulter Counter. Rates of filtration and ingestion were calculated using the equations of Frost (1972), with cell number and ^{35}S as biomass indices.

RESULTS AND DISCUSSION

Changes in T. weissflogii cell number and ^{35}S radioactivity due to grazing by T. longicornis are shown in

Table B1. Of the three experimental bottles, the most cells were grazed from #2 as reflected by three independent measurements: cell number, ^{35}S in the cells remaining in the grazed culture medium, and ^{35}S accumulated in T. longicornis. When this data is applied to the equations of Frost (1972), cell number and ^{35}S give similar grazing results (Table B2). The calculated filtration rates are of the same order of magnitude as the mean value of 0.22 ml^{-1} copepod^{-1} h^{-1} found by Berner (1962) for Temora longicornis grazing on cultures of Skeletonema costatum labelled with ^{32}P.

From the measured number of cells removed from the medium due to grazing, and the mean measured ratio of 3.9 d.p.m. per 10^3 cells, I calculated the radioactivity the zooplankton would contain if they assimilated all of the phytoplankton removed. This calculation gives a measure of "ingestion" by T. longicornis. The actual ^{35}S in the copepod is a measure of "assimilation", assuming that the radioactivity due to unassimilated food in the copepod's gut is negligible. Calculated ingestion was about twice the measured assimilation, giving a mean assimilation efficiency of 56.9% ± 6.2 (Table B3), a value consistent with other studies (Tranter, 1976).

Table B1. Cell concentration and radioactivity remaining after *Temora longicornis* had grazed on ^{35}S-labelled *Thalassiosira weissflogii*.

Bottle	Animals per bottle	Cell concentration (cell number ml^{-1})$\times 10^3$			Radioactivity		
		before	after	difference	d.p.m. after grazing	d.p.m. 10^3 cells	d.p.m. 5 *Temora*
Control	0	5.21	5.70	+0.49	23.6	4.1	-
1	5	5.21	3.84	-1.37	14.7	3.8	451.4
2	5	5.21	3.28	-1.93	11.3	3.4	553.0
3	5	5.21	3.88	-1.33	16.9	4.4	476.5

Table B2. Comparison in three experimental bottles of grazing parameters calculated from cell number (A) and cellular ^{35}S content (B). Temora longicornis fed on ^{35}S-labelled T. weissflogii.

Bottle	Method of calculation	Growth constant k (h^{-1})	Grazing coefficient gr (h^{-1})	Filtration rate F (ml^{-1} copepod^{-1} h^{-1})	Mean cell concentration <C> (cells ml^{-1})	Ingestion rate I (cells copepod^{-1} h^{-1})
1	A	0.0037	.013	0.381	4687	1788
	B	0.0045	.015	0.458	4795*	2181*
2	A	0.0037	.019	0.578	4349	2516
	B	0.0045	.026	0.786	4231*	3314*
3	A	0.0037	.012	0.368	4711	1736
	B	0.0045	.010	0.283	5103*	1441*

A = Calculated from cell number

A = Calculated from ^{35}S content

* = Based on a ratio of 3.9 d.p.m. per 10^3 cells

Table B3. Calculation of the assimilation efficiency of T. longicornis feeding on ^{35}S-labelled T. weissflogii. "Assimilation" is the measured ^{35}S content (d.p.m.) of T. longicornis, and "Ingestion" is the ^{35}S content (d.p.m.) of T. longicornis calculated from the total number of phytoplankon cells removed due to grazing by the copepod. A ratio of 3.9 d.p.m. per 10^3 cells, determined from Table B1, was used to convert cell number to d.p.m.

Bottle #	"Assimilation" (d.p.m.)	"Ingestion" (d.p.m.)	Assimilation Efficiency (%)
1	451.4	783.0	57.7
2	553.0	1100.0	50.3
3	476.5	760.0	62.7

The ^{35}S method for measuring grazing rates is applicable to field situations if labelled cultures or labelled natural phytoplankton populations are used as food organisms. An initial and final independent measure of algal biomass (e.g., chlorophyll a, carbon) is necessary to convert ^{35}S to biomass units for the calculation of ingestion rate but not of filtration rate. The method was tested in the Davis Strait by M. Huntley. It was found, however, that heat-killed zooplankton in the control bottles absorbed more radioactivity from $^{35}SO_4^{2-}$ in the incubation medium than was accumulated by live animals grazing on ^{35}S-labelled phytoplankton in the experimental bottles. Another type of control must therefore be developed.

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