An immunoprobe to measure Rubisco concentrations and maximal photosynthetic rates of individual phytoplankton cells

Mónica V. Orellana and Mary Jane Perry
School of Oceanography, WB-10, University of Washington, Seattle 98195

Abstract

The cross-reactivity of an immunological probe to the key photosynthetic enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) was characterized as part of a larger effort to determine maximal photosynthetic rates of individual phytoplankton cells. Polyclonal antiserum was produced against purified Rubisco from the marine diatom Chaetoceros gracilis. The results of western immunoblotting demonstrated that the antiserum reacted positively with Rubisco from 38 species of algae and higher plants and failed to react with only three species of dinoflagellates and one prochlorophyte species. However, the binding affinity or the strength of the cross-reaction for the polyclonal antiserum with purified Rubisco varied among species. The antiserum was then affinity purified against spinach Rubisco and its binding affinity for purified Rubisco determined by ELISA. Two taxonomic groupings resulted: one with high-binding affinity (these species included chrysophytes, bacillariophytes, prymnesiophytes, and chlorophytes) and the other with low-binding affinity (dinophytes and cyanophytes). Rubisco concentration per cell and light-saturated rates of photosynthesis were highly correlated for cultures of the diatom Thalassiosira weissflogii. These results indicate that affinity-purified antiserum can be rigorously characterized for use in quantifying Rubisco concentration and for assessing the maximal photosynthetic potential of individual phytoplankton cells.

Assessing the magnitude and variance of primary production in the marine environment is a major goal of biological oceanography. Variability in rates of photosynthesis have been observed on all spatial scales from that of the individual phytoplankton cell to the global ocean (Watt 1971; Platt and Sathyendranath 1988) and on all temporal scales from picoseconds to geological epochs (Kolber et al. 1988; Barnola et al. 1987). Understanding the causes of variability in primary production is important if we are to predict nonlinear responses of marine food webs to environmental perturbations or to develop realistic models of the biogeochemical flux of carbon in the ocean. Achievement of that goal requires studies of photosynthesis on a diversity of spatial and temporal scales.

Photosynthetic C fixation has been measured by NaH\(^{14}\)CO\(_3\) incorporation (Steeemann Nielsen 1952) for 40 yr. As generally applied, the \(^{14}\)C method determines C assimilation rates for "bulk" phytoplankton in mixed-species assemblages but gives little or no information about the variability in photosynthetic activity at the scale of the individual species or cell. Statistical information on the underlying variability at the level of the individual phytoplankton cell is necessary to resolve differences in primary production and loss at the next highest scale (i.e. bulk assemblages). Sampling at the scale of the individual cell provides the most basic information for interpreting variance in primary productivity among bulk assemblages (Huston et al. 1988).

A limited number of oceanographic studies of photosynthetic or growth rates of individual phytoplankton cells or species have been carried out with autoradiography (Iturriaga and Marra 1988), \(^{14}\)C labeling of individual cells (Rivkin 1990), and cell division patterns (Weiler and Eppley 1979). Although the results of these studies demonstrate significant interspecific differences in rates, relatively few cells can be processed...
Immunoprobe for Rubisco

because single-cell methods are extremely labor intensive and, hence, limited in their general applicability to field studies of primary productivity. However, with the application of flow cytometry to oceanography, it is possible to analyze autofluorescent and light-scattering properties for thousands of individual cells in a very short time (e.g. minutes). By coupling flow cytometry with immunological probes for taxonomic identification (Shapiro et al. 1989) or quantification of specific cellular components (Ornellas et al. 1988), it is possible to address questions relating to the variability in photosynthetic rates and physiological processes among individual cells or taxonomic groups in phytoplankton assemblages.

The specific goal of the present work was to develop a method to determine maximal photosynthetic potential for individual phytoplankton cells. To do so, we developed an immunological probe against the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The reasons for selecting this key enzyme of the Calvin-Benson cycle are as follows. Rubisco is responsible for net CO2 assimilation. Under conditions which favor carboxylase activity as opposed to oxygenase activity (e.g. high ratios of CO2 to O2), maximal light-saturated rates of photosynthesis are proportional to Rubisco concentrations (Bjorkman 1981; Rivkin 1990). The enzyme is ubiquitous in all photoautotrophic plants and is also found in various photosynthetic bacteria. The large subunit of Rubisco contains the active catalytic site and is highly conserved (Miziorko and Lorimer 1983). Because it is a soluble protein and a major constituent of phytoplankton cells, Rubisco can be easily detected in individual cells with immunofluorescent probes.

In order to use an immunoprobe to quantitatively measure Rubisco concentration within individual cells in a mixed-species assemblage, it is imperative to determine: first, whether the probe is truly specific to only the large subunit of Rubisco; second, whether the probe binds to the Rubisco large subunit of all phytoplankton species with equal affinity; and third, whether immunologically determined concentrations of Rubisco are well correlated with maximal photosynthetic rates. In this paper, we demonstrate that these criteria were satisfied. The results indicate that an affinity-purified anti-Rubisco probe can be used to quantify Rubisco concentrations and maximal photosynthetic potential of individual phytoplankton cells in mixed-species assemblages.

Materials and methods

Cell culture— Cultures of 38 species of algae were grown at 20°C; the other two algal species, *Gonyaulax tamarensis* and *Peridinium balticum*, were grown at 15°C and 28°C. Most species were grown in the light under a 12:12 L/D regime; *G. tamarensis* and *Porphyra yezoensis* were grown under regimes with 18:6 and 16:8 L/D. All diatom cultures and cultures of seven additional species (*Synechococcus bacillaris, Dunaliella tertiolecta, Dunaliella salina, Tetraselminus suecica, Cryptomonas profunda, Isochrysis galbana, and Ochromonas danica*) were grown in modified-IMR media (Perry et al. 1981) at 300 μmol photons m⁻² s⁻¹. *Chlamydomonas moewusii*, *Porphyridium purpureum*, and *Cyanophora paradoxa* were grown in 3 N⁺ medium (Hoham 1971) at 300, 220, and 100 μmol photons m⁻² s⁻¹. *Anabaeana cylindrica* was grown in CCAP media (George 1976) at 100 μmol photons m⁻² s⁻¹ and *Monodus* sp. in ASP2 (Hedberg 1983) at 300 μmol photons m⁻² s⁻¹. The remaining chlorophytes, dinophytes, and phaeophytes were grown in modified f/2 medium (Waaland and Watson 1980) at 150 or 300 μmol photons m⁻² s⁻¹. A lysate of *Prochloron* sp. was obtained from R. A. Cattonico. Rubisco from *Spinacea oleracea* was purified from partially purified Rubisco (Sigma). The two heterotrophic species were obtained from G. Kenny.

Whole-cell lysates—Cells in the logarithmic growth phase were harvested by centrifugation. They were resuspended in 15–40 ml of Rubisco buffer (25 mM Tris-HCl, pH 8.1, 10 mM MgCl₂, 10 mM KCl, 1 mM DTT (dithiothreitol), 0.5 mM EGTA [ethylene glycol-bis(b-amino-ethyl ether)-N,N’-tetraacetic acid], 0.4 mM PMSF (phenylmethylsulfonylfluoride), 0.7 μg ml⁻¹ leu-
peptin, 0.5 \mu g \text{ml}^{-1} \text{pepsatin}, \text{and broken at} 1,155 \text{kg m}^{-2} \text{with a French pressure cell.}

**Rubisco purification**—Purified enzyme was prepared according to Newman and Cattolico (1987a). Whole-cell lysates were centrifuged at 15,000 \times g for 15 min. The supernatant was adjusted to 55\% (v/v) (NH₄)₂SO₄. The resultant precipitate was collected by centrifugation at 18,000 \times g for 15 min and resuspended in 2 ml of Rubisco buffer. The resuspension was loaded onto a 10–40\% linear sucrose gradient prepared in Rubisco buffer and centrifuged at 184,000 \times g for 140 min. Absorbance was monitored at 280 nm for each fraction.

Rubisco identity was confirmed by a 14C-labeling activity assay (Newman and Cattolico 1987a); fractions containing Rubisco were pooled and stored at -40\°C.

**Polyclonal antiserum production**—Rubisco holoenzyme was purified from the diatom *Chaetoceros gracilis* and the antiserum produced in a New Zealand rabbit according to Vaitukaitis (1981). Preimmune serum was obtained before initiation of the injection series. The antiserum was prepared according to Hurn and Chantler (1980) and stored at -40\°C.

**Affinity purification of polyclonal antiserum**—Spinach Rubisco (Sigma R-8000) was coupled to a cyanogen bromide-activated sepharose column (Sigma C-9142) and cross-linked with 1\% glutaraldehyde in PBS (10 mM Na₂HPO₄, pH 7.2, 0.15 M NaCl) for 1 h. The antiserum was loaded onto the column and the column washed with PBS. Antibodies that cross-reacted with domains on spinach Rubisco were linked to the spinach-sepharose column, while antibodies that did not recognize domains on spinach Rubisco were eluted and discarded. The reactive antibodies were eluted with 100 mM glycine, pH 3.2; these affinity-purified monospecific antibodies were washed with PBS and stored at -40\°C.

**Electrophoresis**—Cell lysates or purified Rubisco samples were diluted 1:2 in sample buffer containing 0.5 M Tris HCl, pH 6.8, 10\% (v/v) glycerol, 2\% (w/v) SDS, and 5\% (v/v) 2-\beta-mercaptoethanol. The samples were heated for 4 min at 95\°C. Discontinuous SDS-PAGE gel electrophoresis was carried out according to Laemmli (1970) with 25 mA at room temperature. The gels were stained with 0.1\% Coomassie Brilliant Blue R-250 in 40\% methanol (v/v) with acetic acid (10\% v/v), destained, and scanned with a laser densitometer (Helena) for estimation of protein in the gel bands.

**Western blots**—The separated proteins on the gels were electrophoretically transferred to nitrocellulose membrane overnight according to Towbin et al. (1979). After electrotransfer, the membrane was blocked with 2\% BSA (bovine serum albumin) in TBS (Tris-buffered saline; 20 mM Tris-HCl, pH 7.5, 500 mM NaCl), washed in TBS, and incubated in primary (anti-Rubisco) antiserum diluted to 1:1,000 with 2\% BSA in TTBS (0.05\% Tween 20 in TBS) for 2 h. The membrane was washed in TTBS and incubated in secondary antibody (BioRad goat anti-rabbit IgG conjugated with horseradish peroxidase). The immunoprecipitate was visualized by incubating the membrane in substrate solution [0.6 mg ml⁻¹ 4-chloro-1-naphthol in methanol, 8 ml of PBS, and 0.1\% H₂O₂ (v/v)]. The reaction was terminated with deionized water.

**Dot blot**—A series of concentrations from 5 to 1,000 ng of purified Rubisco was loaded onto nitrocellulose membrane according to Moeremans et al. (1984). The immunobinding and detection were carried out as described for the western immunoblotting.

**ELISA assay**—Purified Rubisco, in concentrations ranging from 24 to 500 ng, was diluted in 0.1 M carbonate buffer (0.1 M NaHCO₃, pH 9.6, 10 mM MgCl₂) and immobilized in triplicate wells of Costar No. 3590 microtiter plates by passive attachment during overnight incubation at 4\°C according to Catt and Millard (1988) with modifications. The plates were washed in PBS, incubated in a blocking buffer (2\% BSA in PBS), reashed in PBS-T (PBS with 0.05\% Tween 20), and incubated with affinity-purified antiserum, diluted to 1:100 in PBS-T with 2\% BSA, for exactly 2 h at room temperature. The plates were washed in PBS-T and incubated with secondary antibody (BioRad goat antirabbit IgG conjugated with alkaline phosphatase, diluted 1:3,000 in 2\% BSA in PBS-T) for 2 h. The plates were washed and incubated in substrate solution (4-nitrophenyl phosphate in...
0.1 M diethanolamine buffer, pH 9.8, 1 mM MgCl₂). The reaction was terminated with NaOH (15 mM), and optical density at 405 nm was measured with an ELISA plate reader (Cambridge Technology). The linear portions of the hyperbolic ELISA-binding curves were analyzed by analysis of variance within species and analysis of homogeneity of slope regressions among species.

Immunofluorescent staining - The procedure consists of three steps: fixation, permeabilization, and Rubisco immunofluorescent staining. Cells were harvested in a refrigerated centrifuge at 500 × g and 4°C for 10 min and subsequently fixed in freshly prepared 2% paraformaldehyde in PBS pH 8.0 at 4°C for 10 min. Cells were permeabilized in 0.5% DMSO (dimethylsulfoxide) in PBS for 3–5 min on ice. Tests for potential leakage of Rubisco from the cells during fixation and permeabilization were carried out by dot blot immunoassay (Moeremans et al. 1984). Because paraformaldehyde cross-links this soluble enzyme and prevents its leakage from the cell (Ohba et al. 1979), no Rubisco was found in the supernatant. Although paraformaldehyde changes enzyme secondary structure, no loss of antigenicity for Rubisco was observed.

The cells were stained by first incubating them on ice with affinity-purified Rubisco antiserum diluted (1 : 100) in PBS with 2% BSA. After 2 h, the cells were washed with 15 ml of PBS and then incubated with a goat anti-rabbit secondary antibody conjugated with FITC (fluorescein isothiocyanate) at a 1 : 300 dilution for 1 h. The cells were washed three times for 5 min in 15 ml of PBS. Three controls were run at the same time on fixed and permeabilized cells to evaluate nonspecific staining: cells without either primary or secondary antisera, cells incubated in preimmune serum and secondary antiserum, and cells incubated in secondary antiserum alone. The fixation and staining procedures were evaluated qualitatively by epifluorescence microscopic observations.

Flow cytometric analysis - The fluorescence of individual cells was quantified with a Coulter Epics Profile flow cytometer with an argon laser tuned to 488 nm and operated at 15 mW. The Epics Profile was calibrated with "Immuno-Check" fluorospheres (Coulter Corp.). Short-pass (530 nm) and long-pass (630 nm) filters were used to detect the green and red fluorescence from FITC and Chl a, respectively. Forward-angle scatter data also were collected to test cell integrity. Fluorescence and light scatter data were recorded on a 3-decade log₁₀ scale, stored as one- or two-parameter histograms, and transformed to a linear scale for analysis. The magnitude of Rubisco immunostaining was calculated as the difference between the green fluorescence of the primary plus secondary immunostained cells and that of the control cells stained with only secondary antiserum.

Photosynthetic measurements - Photosynthetic rate measurements were determined for cultures of the diatom Thalassiosira weissflogii grown on a 15 : 9 L/D cycle at 5, 50, 100, 150, or 300 μmol photons m⁻² s⁻¹ at 20°C. Irradiance was measured with a quantum scalar irradiance meter (Biophotential QSL-100). Cultures were adapted to their growth irradiance for 3 weeks before initiation of experiments. They were maintained at low cell densities in logarithmic growth by diluting the cultures every other day with fresh media to avoid self-shading and to ensure nonlimiting nutrient concentrations. Specific growth rates (d⁻¹) were determined from cell counts. Chl a was measured fluorometrically (Lorenzen 1966).

Photosynthetic rates were measured by NaH¹⁴CO₃ incorporation in P vs. I (photosynthesis vs. irradiance) incubators with 48 irradiances, ranging from 3.7 to 1,525 μmol photons m⁻² s⁻¹ at 20°C (Talbot et al. 1985). One-milliliter aliquots were incubated for 0.5 h at 20°C with 0.06 μCi ¹⁴C ml⁻¹; total activity was determined with PEA (phenoxyethanolamine; Iverson et al. 1976). The incubation was terminated by adding 250 μl of 6 N HCl; samples were shaken for 1 h to remove excess ¹⁴CO₂. The P vs. I data were fit to a nonlinear exponential equation to derive the photosynthetic parameter Pₘₐₓ (light-saturated photosynthetic rate).

Results

Polyclonal antiserum - Cross-reactivity of the anti-Rubisco polyclonal antiserum with Rubisco from 44 species of autotrophs
Table 1. Cross-reactivity of anti-Rubisco antiserum produced against purified Rubisco from *Chaetoceros gracilis*. Cross-reactions—(+); failure of antiserum to cross-react.—(−).

| Species                        | Cross-reactivity | No. of analyses |
|--------------------------------|------------------|-----------------|
| **Cyanophyta**                 |                  |                 |
| *Synechococcus bacillaris* Butcher | +                | 2               |
| *Cyanophora paradoxa* Korshikov |                  |                 |
| *Anabaena cylindrica* Lemmermann | +                | 1               |
| **Prochlorophyta**             |                  |                 |
| *Prochloron* sp.               | −                | 2               |
| **Cryptophyta**                |                  |                 |
| *Cryptomonas profunda* Butcher* | +                | 1               |
| **Prymnesiophyta**             |                  |                 |
| *Isochrysis galbana* Parke*     | +                | 4               |
| **Chrysophyta**                |                  |                 |
| *Ochromonas danica* Pringsheim* |                  |                 |
| *Olisthodiscus luteus* N. Carter*† | +              | 8               |
| **Eustigmatophyta**            |                  |                 |
| *Monodus* sp.*                 | +                | 1               |
| **Bacillariophyta**            |                  |                 |
| *Chaetoceros gracilis* Schuett* |                  |                 |
| *Cylindrotheca fusiformis* Reimann et Lewin* | +    | 4               |
| *Phaeodactylum tricornutum* Bohlin* | +            | 4               |
| *Thalassiosira weissflogii* (Grunow) Fryxell et Hasle* | +   | 8               |
| *Nitzschia closterium* (Ehrenberg) W. Smith | + | 4               |
| *Ditylum brightwelli* (West) Grunow‡ | + | 2               |
| **Dinofyta**                   |                  |                 |
| *Amphidinium carterae* Hulburt* | −                | 2               |
| *Amphidinium klebsii* Kofoid et Swezy | +        | 4               |
| *Cachonella ildefina* Herman et Sweccny | +   | 4               |
| *Glenodinium* sp.              | +                | 4               |
| *Gonyaulax catenella* (Levander) Kofoid | −   | 2               |
| *Gonyaulax tamarensis* Lebour*§ | +            | 3               |
| *Gymnodinium simplex* (Lohmann) Kofoid et Swezy | +   | 2               |
| *Heterocapsa niei* (Loeblich III) Morrell et Loeblich | +   | 2               |
| *Peridinium balticum* (Levander) Lemmermann | +   | 2               |
| *Wolosynskia limnetica* Bursa | +                | 4               |
| **Chlorophyta**                |                  |                 |
| *Chlamydomonas moewusii* (Brown et al.)* | +    | 2               |
| *Dunaliella salina* (Dunal) Teodoresco* | +    | 4               |
| *Dunaliella tertiolecta* Butcher‡ | +        | 2               |
| *Tetraselmis suecica* Kylin     | +                | 2               |
| *Valonia ventricosa* J. Agardh | +                | 2               |
| *Siphonocladus* sp.            | +                | 2               |
| *Batophora verstelii* J. Agardh | +             | 2               |
| *Cladophoropsis membranacea* (C. Agardh) Borgeso | +    | 2               |
| *Bryopsis hypnoides* Lamoureux | +             | 2               |
| **Euglenophyta**               |                  |                 |
| *Eutreptiella marina* Da Cuhna | +                | 4               |
| **Phaeophyta**                 |                  |                 |
| *Dictyota dichotoma* (Hudson) Lamoureux | +    | 2               |
| **Rhodophyta**                 |                  |                 |
| *Griffithsia pacifica* Kylin*   | +                | 3               |
| *Porphyra yeoensis* Ueda*       | +                | 3               |
| *Botryocladia uvatiodes* Dawson | +             | 2               |
| *Spiridium filamentosa* (Wulf) Harvey | +   | 2               |
| *Porphyridium purpureum* (Bory) Drew et Ross | +  | 2               |
Table 1. Continued.

| Species                     | Cross-reactivity | No. of analyses |
|-----------------------------|------------------|----------------|
| Angiospermae                |                  |                |
| *Spinacea oleracea* Linne*  | -                | 8              |
| Heterotrophic protozoans    |                  |                |
| *Trichomonas vaginalis*‡    |                  | 3              |
| *Giardia lamblia*‡          |                  | 3              |

* Purified Rubisco.
† R. A. Cattolico clone.
‡ Immunofluorescent staining with epifluorescent microscopic observation of whole cells.
§ Purified Rubisco reacted (+), but whole cell lysate did not react (−).

(representing 14 groups of microalgae, macroalgae, and one higher plant) was analyzed by western blot analysis. In addition, whole-cell lysates from two heterotrophic species were analyzed. Most species were analyzed a minimum of 2 times. Almost all the species cross-reacted with the probe, except for the one species of prochlorophyte, the two heterotrophic protozoans, and three species of dinoflagellates. A fourth dinoflagellate, *G. tamarensis*, exhibited positive cross-reactivity against its purified Rubisco but failed to cross-react when its whole-cell lysate was tested. In almost all cases where cross-reactivity was observed only one cross-reacting band, representing the large subunit of the Rubisco holoenzyme, was found in whole-cell lysates. The results from these western blots are summarized in Table 1 and demonstrate specificity of the antiserum for the Rubisco large subunit as well as a pattern of broad interspecific cross-reactivity. However, western blots are strictly a qualitative indicator of the presence or absence of antibody-antigen cross-reactivity and are not a quantitative index of the strength of the cross-reaction. When the more quantitative dot blot assays were used with purified Rubisco from different species, a high degree of variability in the binding affinity with the polyclonal antiserum was evident (Fig. 1). As a consequence of this interspecific heterogeneity in the strength of the antibody-antigen binding, it was necessary to affinity purify the polyclonal antiserum.

**Affinity-purified antiserum**—For whole-cell lysates, the Rubisco large subunit was the only band evident in the SDS-PAGE gel (Fig. 2, left) which cross-reacted with the antiserum in the western blot (Fig. 2, right), demonstrating the high specificity of the antiserum for the Rubisco large subunit. The affinity-purified probe cross-reacted with the Rubisco large subunit (Fig. 2, right), confirming that no unexpected alteration occurred in probe specificity due to affinity purification.

The binding capacity or strength of the antibody-antigen interaction between purified Rubisco and the affinity-purified antiserum was quantified by ELISA assay. The results of the assay were highly reproducible, with an average SE of 0.192 for all points on the curves. Variability between

---

Fig. 1. Dot blot analysis to measure cross-reaction between polyclonal Rubisco antiserum and nanogram concentrations of purified Rubisco from: a—*Olisthodiscus luteus*; b—*Isochrysis galbana*; c—*Chaetoceros gracilis*; d—*Phaeodactylum tricornutum*; e—*Porphyra yezoensis*; f—*Tetraselmis suecica*; g—*Chlamydomonas moewusii*. 
assays run on different days was low; results of two independent experiments with purified Rubisco from *I. galbana* are shown in Fig. 3. No statistically significant difference was found between the slopes for the two experiments (*P* ≤ 0.001). When purified Rubisco from 11 species representing different systematic groups was analyzed by ELISA, two distinct groupings were evident (Table 2). Group 1 had a high binding affinity for the affinity-purified antiserum and included species representing the chrysophytes, prymnesiophytes, bacillariophytes, chlorophytes, and rhodophytes. It should be noted that the binding affinity for the affinity-purified antiserum to Rubisco, purified from the immunizing species *C. gracilis* was greater than for the other species in group 1 and that rhodophyte species had a lower affinity. The binding affinity in group 2 was much lower; the second group of species included the cyanophyte *Synechococcus bacillaris* and the dinophytes *Glenodinium* sp., *Gymnodinium simplex*, and *Amphidinium carterae*.

Rubisco concentration and the light-saturated rate of photosynthesis—The growth rate of *T. weissflogii* ranged from 0.09 d⁻¹ at 5 μmol photons m⁻² s⁻¹ to 1.06 d⁻¹ at 300 μmol photons m⁻² s⁻¹; however, growth was not saturated at the highest irradiance. The concentration of Chl *a* cell⁻¹ decreased by a factor of 2.9 as growth irradiance increased from the lowest to the highest irradiance, while the cellular absorption cross-section estimated from flow cytometric Chl *a* fluorescence cell⁻¹ according to Perry and Porter (1989) changed by a factor of 1.9 over the growth irradiances examined. Light-saturated rates of photosynthesis per cell increased by a factor of 4.4 as a function of increasing growth irradiance.

Figure 4 shows an example of *T. weissflogii* cells grown at 25 μmol photons m⁻² s⁻¹ that were immunostained with the anti-Rubisco probe. The cells were analyzed flow cytometrically and displayed on a 3-decade log₁₀ scale. The control cells were fixed, permeabilized, and stained with only the sec-
Table 2. ELISA assays with affinity-purified anti-Rubisco antiserum and purified Rubisco protein. Maximal optical density at 405 nm—Max OD. Slope and $r^2$ (of slope) are for ELISA hyperbolic curves.

| Species                | Group 1                              | Group 2                             |
|------------------------|--------------------------------------|-------------------------------------|
|                        | Max OD  | Slope     | $r^2$  | Max OD  | Slope     | $r^2$  |
| Chaetoceros gracilis   | 10.5    | 0.123±0.009 | 0.96   | 0.9     | 0.003±0.002 | 0.75   |
| Phaeodactylum tricornutum | 9.0    | 0.075±0.005 | 0.95   | 0.9     | 0.003±0.002 | 0.74   |
| Isochrysis galbana (1) | 9.5     | 0.099±0.007 | 0.96   | 0.9     | 0.002±0.002 | 0.78   |
| Olisthodiscus luteus   | 9.5     | 0.103±0.001 | 0.91   | 0.9     | 0.001±0.0007 | 0.68   |
| Dunaliella salina      | 9.0     | 0.080±0.006 | 0.96   | 0.9     | 0.001±0.0007 | 0.68   |
| Spinacea oleracea      | 9.0     | 0.067±0.003 | 0.96   | 0.9     | 0.001±0.0007 | 0.68   |
| Porphyra yezoensis     | 7.1     | 0.047±0.005 | 0.96   | 0.9     | 0.001±0.0007 | 0.68   |

They exhibited very low background green fluorescence relative to the Rubisco-stained cells. The geometric mean in relative fluorescence units, converted to linear units from log$_{10}$ units, was 3 for the control cells while the geometric mean for the immunostained cells was 172.5. The background fluorescence of the control cells included a component due to native green autofluorescence (Shapiro 1988) plus a component due to nonspecific staining by the secondary goat antirabbit antibody. In general the total fluorescence of the control cells was low, averaging between 3 and 8% of the signal for the Rubisco-stained cells. Figure 5 shows the regression ($r^2 = 0.90$) of the concentration of Rubisco per cell determined by immunofluorescence against $P_{\text{max}}$ per cell.

**Discussion**

**Probe characterization**—If an immunological probe is to be used to quantify Rubis-
co concentration in individual phytoplankton cells in a mixed-species assemblage, it must meet the following criteria: cross-react with only the Rubisco large subunit; bind quantitatively to the Rubisco large subunit; and have equal affinity for the Rubisco large subunit from all species of marine phytoplankton. The affinity-purified monoclonal polyclonal antiserum reported here meets these criteria for group 1 species. One reason that it was possible to select for a species-independent Rubisco probe is that the large subunit protein is highly conserved in an evolutionary context (Miziorko and Lorimer 1983). Immunological cross-reactivity (Table 1), hybrid reconstitution (Andrews and Lorimer 1987), heterologous hybridization (Shively et al. 1986), and amino acid sequences (Miziorko and Lorimer 1983) all suggest that the large subunit of higher plants, green algae, and cyanobacteria is conservative, particularly at the active sites. Taxa-specific differences have been reported for other anti-Rubisco antisera (Plumley et al. 1986; Newman and Cattolico 1987b); it should be noted that these polyclonal antisera were not affinity purified to conservative sites. In contrast, in the present study the antiserum was affinity purified to Rubisco from a distant species in an effort to select only antibodies to conservative sites.

The data in Table 1 show that for 44 species tested with our *C. gracilis* polyclonal anti-Rubisco antiserum, 38 exhibited positive cross-reactivity. The species that did not cross-react included three species of dinoflagellates (*A. carterae*, *Gonyaulax catenella*, and *Heterocapsa neu*), the one species of prochlorophyte, and the two nonphotosynthetic heterotrophic protozoans. The lack of cross-reactivity with the dinoflagellates is puzzling. It may reflect a low total concentration of Rubisco in the whole-cell lysate, since positive cross-reactivity was detected in a western blot of purified Rubisco from *G. tamarensis* but not in the whole-cell lysate (Table 1). Alternatively, the lack of cross-reactivity could reflect a minimal number of common domains on the large subunit of these dinoflagellate species or degradation of the enzyme during lysate preparation. Coprecipitation of Rubisco with the membrane fraction during centrifugation is also possible, as it has been previously observed that extraction in certain buffers, such as those containing MgCl₂, can result in association of Rubisco with membranes in some species (McNeil and Walker 1981).

The results of the western blot analyses with whole-cell lysates demonstrated a high degree of cross-reactivity across species groups (Table 1), as might be predicted from the conservative structure of the Rubisco large subunit. However, the dot blot analysis with purified Rubisco revealed high variability in the binding affinity of the polyclonal antiserum with the large subunit of different species (Fig. 1). The source of this heterogeneity is probably the presence of a diversity of antibody molecules in the polyclonal antiserum, with each type of antibody having a different affinity or strength of interaction to the different antigenic domains on the Rubisco large subunit (Atassi 1984). Although the active site of the large subunit of Rubisco is highly conserved, some of the antibodies could have been produced against nonconservative sites. Such heterogeneity in the binding affinity would prevent the use of Rubisco probes to quantify Rubisco concentrations in single cells present in a mixed-species assemblage.

This problem was addressed by affinity purification of the polyclonal antiserum against Rubisco from spinach, a species evolutionarily distant from *C. gracilis*. The reasoning is that only a subset of antibodies, i.e. those that react with the most conservative sites on the protein, are selected by binding to the affinity-purification column, while the antibodies that do not recognize domains on spinach Rubisco are eluted and eliminated. The antibodies that do react with domains on spinach Rubisco should be monospecific, and therefore, should theoretically cross-react with the same sites on all phytoplankton species and with the same binding affinity.

These concepts were tested with purified Rubisco. The results presented in Fig. 2 indicate that the antiserum was monospecific and bound only to the Rubisco large subunit. The results presented in Table 2 indicate that for our affinity-purified antiserum the concept of uniform binding affinity
applied only to group 1 species; the implication is that our affinity-purified antiserum can be used only to quantify Rubisco in species of chrysophytes, bacillariophytes, prymnesiophytes, and chlorophytes (group 1). Although the rhodophyte species in group 1 had a slightly lower binding affinity, this deviation is not critical to our application, because this group is not phytoplanktonic. A significantly lower binding affinity was observed for group 2, which consisted of dinophyte and cyanophyte species. The explanation for this pattern, particularly for the dinophytes, is not obvious; however, a comparison of their Rubisco structure might yield interesting evolutionary patterns.

The oceanographic implications of the characterization and standardization of the Rubisco antiserum is that the affinity-purified probe can be used in mixed-species assemblages to quantify Rubisco concentrations in individual phytoplankton cells, particularly for mixed-species assemblages dominated by chrysophytes, bacillariophytes, prymnesiophytes, and chlorophytes. Because the probe has a much lower binding affinity for dinoflagellates and cyanobacteria, Rubisco cannot be measured in these groups and it is essential to identify individuals belonging to these groups. Oceanic coccoid cyanobacteria can be distinguished flow cytometrically by their phycoerythrin fluorescence and characteristic low forward-scatter signal (Olson et al. 1988; Perry and Porter 1989). Dinoflagellates are more problematic; however, cell-surface antibody probes may prove to be sufficient for discriminating dinoflagellates by flow cytometry. The technology for image and fluorescence analysis of immobilized cells is rapidly improving and may also provide a solution for dinoflagellate recognition.

This study demonstrates the importance of immunoprobe standardization. Although Rubisco has been previously quantified with heterologous antisera by a variety of different methods (radioimmunoassay, radial immunodiffusion, rocket immunoelectrophoresis, and dot blots), the strength of the antibody-antigen interaction or affinity of the antiserum for Rubisco purified from different species has not, to our knowledge, been quantified previously. Equal binding affinity of the antiserum with different species has been assumed. Although this may be true when Rubisco from closely related species is being measured, the results of the immunoprobe standardization demonstrate that the assumption is incorrect when Rubisco is measured in an evolutionarily different group of species. The cross-reactivity patterns reported by Plumley et al. (1986) and Newman and Cattolico (1987b) suggest that not all the antibodies in their polyclonal mixtures were against conservative sites, similar to our polyclonal mixture prior to affinity purification. Their findings further confirm the need for rigorous characterization of antisera used in mixed-species applications. Monoclonal antibodies to the active site of Rubisco may provide a more universal solution.

Rubisco concentration and the light-saturated rate of photosynthesis—A strong relationship between maximal extractable Rubisco activity and maximal capacity for photosynthesis has been observed in higher plants (Bjorkman 1981) and in microalgae (Rivkin 1990). Total in vitro Rubisco activity has been correlated with maximal light-saturated rates of photosynthesis in the diatom *Phaeodactylum tricornutum* at low growth irradiance (Beardall and Morris 1976), the diatom *C. gracilis* grown under nitrogen stress (M. J. Perry unpubl.), the green alga *Scenedesmus obliquus* (Senger and Fleischhaker 1978), the dinoflagellate *Pyrocystis noctiluca* (Rivkin et al. 1982), and several other individual species isolated from natural populations (Rivkin 1990). In studies of marine phytoplankton assemblages, total Rubisco activity has also been correlated with light-saturated rates of photosynthesis (Glover and Morris 1979; Smith et al. 1983). However, a number of the past observations of the relationship between \( P_{\text{max}} \) and Rubisco concentration, as determined by in vitro assay activity, have been conflicting. For example, Glover and Morris (1979) and Smith et al. (1983) found that Rubisco activity would not account for 100% of the photosynthetic potential, as a consequence either of partial recovery of enzyme activity or differing physiological responses of individual species within a mixed-species assemblage. This observa-
tion led Glover and Morris to conclude that Rubisco activity was a poor indicator of the light-saturated rates of photosynthesis.

The relationship in the past between in vitro Rubisco activity and $P_{\text{max}}$ has been controversial. First, the early in vitro enzymatic assays resulted in unnaturally low rates of CO$_2$ fixation because the importance of the sequence of substrate addition on Rubisco activation was not appreciated until the mid-1970s (Lorimer 1979). Second, the importance of light activation of Calvin cycle enzymes and the role of specific inhibitors on in vivo photosynthetic rate and in vitro Rubisco activity was not fully recognized until more recently (Ogren et al. 1986). Third, in vivo net CO$_2$ fixation varied greatly in response to changes in the ratio of CO$_2$ to O$_2$ because of the bifunctional nature of Rubisco as both a carboxylase and an oxygenase (Jordan and Ogren 1981). The enormous variation in the ratio between terrestrial and marine ecosystems suggests caution in directly extrapolating results from one system to another.

Relatively few immunological measurements of both Rubisco concentration and photosynthetic rates have been made for microalgae. The results that are available for chlorophyte species show good agreement between Rubisco concentration and $P_{\text{max}}$. Sukenik et al. (1987) measured the Rubisco large subunit in the marine alga Dunaliella tertiolecta with a radioimmunoassay. They reported that $P_{\text{max}}$ per cell was independent of growth irradiance between 80 and 2,000 μmol photons m$^{-2}$ s$^{-1}$. The cellular concentration of the large subunit of Rubisco, measured both immunologically and by in vitro activity assays, exhibited the same pattern as $P_{\text{max}}$. When the freshwater alga Tetraedron minimum was grown at 50 and 500 μmol photons m$^{-2}$ s$^{-1}$, the immunologically determined Rubisco concentration per cell was constant while $P_{\text{max}}$ per cell varied only 25% between the high- and low-light adapted cultures (Fisher et al. 1989).

Oceanographic applications—The ability to measure Rubisco protein and predict maximal photosynthetic rates in individual cells presents many opportunities; its widespread application will depend on the universality of this relationship in phytoplankton. This approach will provide statistical information on the variability in primary production potential among individual phytoplankton cells within mixed-species assemblages. To achieve the main goal of determining the in situ photosynthetic rate of individual cells in the ocean, we need three other parameters in addition to $P_{\text{max}}$ per cell: underwater irradiance, absorption cross-section of the individual cell, and the functional form of the photosynthesis vs. absorption relationship. Spectral underwater irradiance can be readily obtained, while Perry and Porter (1989) have demonstrated a strong correlation between single-cell Chl a fluorescence determined flow cytometrically and the whole-cell absorption cross-section. Theoretical and empirical advances on the functional relationship between photosynthesis and absorption are continuing; by coupling these advances with information on $P_{\text{max}}$ per cell, in situ irradiance, and single-cell absorption it will be possible to model in situ production for individual cells. By combining this approach with taxon-specific probes (Shapiro et al. 1989), it should be possible to assess the contribution by specific taxa to primary productivity.

References

Andrews, T. J., and G. H. Lorimer. 1987. Rubisco: Structure and prospects of improvement, p. 131–218. In P. K. Stumpf and E. E. Conn [eds.], The biochemistry of plants. V. 10. Academic.

Atassi, M. Z. 1984. Immune recognition of proteins, p. 15–51. In M. Z. Atassi et al. [eds.], Molecular immunology. Dekker.

Barnola, J. M., D. Reynaud, Y. S. Korotkevich, and C. Lorius. 1987. Vostok ice core provides 160,000-year record of atmospheric CO$_2$. Nature 329: 408–414.

Beardall, J., and I. Morris. 1976. The concept of light intensity adaptation in marine phytoplankton: Some experiments with Phaeodactylum triicornutum. Mar. Biol. 37: 377–387.

 Bjorkman, O. 1981. Responses to different quantum flux densities, p. 57–107. In O. L. Lange et al. [eds.], Encyclopedia of plant physiology, new series, V. 12A. Springer.

Catt, J. W., and P. Millard. 1988. The measurement of ribulose-1,5-bisphosphate carboxylase/oxygenase concentrations in the leaves of potato plants by enzyme linked immunosorption assays. J. Exp. Bot. 39: 157–165.

Fisher, T., R. Shuritz-Swirski, S. Gepstein, and Z. Dubinsky. 1989. Changes in the levels of ribu-
lose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in *Tetraedron minimum* (Chlorophyta) during light and shade adaptation. Plant Cell Physiol. 30: 221-228.

George, E. A. [ed.]. 1976. Culture centre of algae and protozoa list of strains. Cambridge.

Glover, H. E., AND I. Morris. 1979. Photosynthetic carboxylating enzymes in marine phytoplankton. Limnol. Oceanogr. 24: 510-519.

Hedberg, M. F. 1983. Investigation of the chloroplast genome in two marine algae, *Codium fragile* and *Monodus* sp. Ph.D. thesis, Univ. North Carolina. 75 p.

Hoham, R. W. 1971. Laboratory and field studies on snow algae of the Pacific Northwest. Ph.D. thesis, Univ. Washington. 207 p.

Hurn, B. A. L., AND S. M. Chantler. 1980. Protein identification. Meth. Enzymol. 70: 105-142.

Huston, M., D. DeAngelis, AND W. Post. 1988. New computer models unify ecological theory. BioScience 38: 682-691.

Iturriga, R., AND J. Marra. 1988. Temporal and spatial variability of chroococcoid cyanobacteria *Synechococcus* spp. specific growth rates and their contribution to primary production in the Sargasso Sea. Mar. Ecol. Prog. Ser. 44: 175-181.

Iverson, R. L., H. F. Brittaker, AND V. B. Myers. 1976. Loss of radiocarbon in direct use of aquasol for liquid scintillation counting of solutions containing 14C-NaHCO3. Limnol. Oceanogr. 21: 756-758.

Jordaan, D. B., AND W. L. Ooren. 1981. Species variation in the specificity of ribulose biphosphate carboxylase/oxygenase. Nature 291: 513-515.

Kolber, Z., J. Zehr, AND P. G. Falkowski. 1988. Effects of growth irradiance and nitrogen limitation of photosynthetic energy conversion in photosystem 2. Plant Physiol. 88: 923-929.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lorenzen, C. J. 1966. A method for the continuous measurement of *in vivo* chlorophyll concentration. Deep-Sea Res. 13: 223-227.

Lorimer, G. H. 1979. Evidence for the existence of discrete activator and substrate sites for CO2 on ribulose-1,5-bisphosphate carboxylase. J. Biol. Chem. 254: 5599-5601.

McNeil, P. H., AND D. A. Walker. 1981. The effect of magnesium and other ions on the distribution of ribulose-1,5-bisphosphate in chloroplast extracts. Arch. Biochem. Biophys. 208: 184-188.

Miziorko, H. M., AND G. H. Lorimer. 1983. Ribulose-1,5-bisphosphate carboxylase-oxygenase. Annu. Rev. Biochem. 52: 507-545.

Moeremans, G., A. Daniels, A. Van Driek, G. Langanger, AND J. De May. 1984. Sensitive visualization of antigen-antibody reactions in dot and bioimmuno overlay assays with immunogold and immunogold/silver staining. J. Immunol. Meth. 74: 353-360.

Newman, S. M., AND R. A. Cattolico. 1987a. Structural, functional and evolutionary analysis of ribulose-1,5-bisphosphate carboxylase from the chromophytic alga *Olisthodiscus luteus*. Plant Physiol. 84: 483-490.

Newman, S. M., AND R. A. Cattolico. 1987b. Structural and functional relatedness of chromophytic and rhodophytic RuBP carboxylase enzymes. p. 671-674. In R. B. Biggins [ed.], Progress in photosynthesis research. Martinus Nijhoff.

Oorena, W. L., M. E. Salvucci, AND A. R. Fortis. 1986. The regulation of Rubisco activity. Phil. Trans. R. Soc. Lond. Ser. B 313: 337-346.

Ohba, Y., Y. Morimitsu, AND A. Watara. 1979. Reaction of formaldehyde with calf thyminus nucleohistone. Eur. J. Biochem. 100: 285-293.

Olson, R. J., S. W. Chisholm, E. R. Zettler, AND E. V. Ambrust. 1988. Analysis of *Synechococcus* pigment types in the sea using single and dual beam cytometry. Deep-Sea Res. 35: 425-440.

Orellana, M. V., M. J. Perry, AND B. A. Watson. 1988. Probes for assessing single-cell primary production: Antibodies against ribulose-1,5-bisphosphate carboxylase (RuBPCase) and peridinin/chlorophyll a protein (PCP). p. 243-262. In C. M. Yentsch et al. [eds.], Immunological approaches to coastal, estuarine, and oceanographic questions. Springer.

Perry, M. J., AND S. M. Porter. 1989. Determination of the cross-section absorption coefficient of individual phytoplankton cells by analytical flow cytometry. Limnol. Oceanogr. 34: 1727-1738.

Platt, T., AND S. Sathyendranath. 1988. Oceanic primary production: Estimation by remote sensing at local and regional scales. Science 241: 1613-1620.

Plumley, F. G., D. L. Kirchman, R. E. Hodson, AND G. W. Schmidt. 1986. Ribulose biphosphate carboxylase from three chlorophyll c-containing algae: Physical and immunological characterization. Plant Physiol. 80: 685-691.

Rivkin, R. B. 1990. Photoadaptation in marine phytoplankton: Variations in ribulose-1,5-bisphosphate carboxylase activity. Mar. Ecol. Prog. Ser. 62: 61-72.

Shapiro, H. M. 1988. Practical flow cytometry. Liss.

Shapiro, L. P., L. C. Campbell, AND E. Haugan. 1989. Immunochemical characterization of ultraplankton species. Mar. Ecol. Prog. Ser. 57: 219-224.

Shively, J. M., AND OTHERS. 1986. Molecular evolution of the large subunit of ribulose-1,5-bisphosphate/oxygenase Rubisco. FEBS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 37: 251-257.
SMITH, J. C., T. PLATT, AND W. G. HARRISON. 1983. Photoadaptation of carboxylating enzymes and photosynthesis during spring bloom. Prog. Oceanogr. 34: 1524–1544.

STEENMAN NIELSEN, E. 1952. The use of radioactive carbon (C\textsuperscript{14}) for measuring organic production in the sea. J. Cons. Cons. Int. Explor. Mer. 18: 117–140.

SUKENIK, A., J. BENNET, AND P. G. FALKOWSKI. 1987. Light-saturated photosynthesis limitation by electron transport or carbon fixation? Biochim. Biophys. Acta 891: 205–215.

TALBOT, M. C., D. S. THORESON, AND M. J. PERRY. 1985. Photosynthesis vs. light intensity measurements: A miniaturized incubator. Univ. Wash. ONR Tech. Rep. 406. Ref. M85-9.

TOWBIN, M. A., T. STAHELIN, AND J. GORDON. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. 76: 4350–4354.

VAITUKAITIS, J. L. 1981. Production of antisera with small doses of immunogen: Multiple intradermal injections. Meth. Enzymol. 73: 46–75.

WAALAND, S. D., AND B. A. WATSON. 1980. Isolation of a cell-fusion hormone from Griffithsia pacifica Kylin, a red alga. Planta 149: 493–497.

WATT, D. W. 1971. Measuring the primary production rates of individual phytoplankton species in natural mixed populations. Deep-Sea Res. 18: 329–339.

WEILER, C. S., AND R. W. EPPLEY. 1979. Temporal patterns of division in the dinoflagellate genus Ceratium and its application to the determination of growth rate. J. Exp. Mar. Biol. 39: 1–24.

Submitted: 18 April 1991
Accepted: 12 November 1991
Revised: 3 February 1992