ANALYSIS OF CHLOROPHYLL FLUORESCENCE

IN MARINE PHYTOPLANKTON:

INTERPRETATION OF FLOW CYTOMETRIC SIGNALS

by

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ABSTRACT

The relationship between flow cytometry fluorescence signals and photosynthetic pigments was investigated in three species of marine eukaryotic phytoplankton, *Thalassiosira weissflogii*, *Hymenomonas carterae*, and *Amphidinium carteri*. Fluorescence was observed to increase linearly with non-zero intercept as a function of chlorophyll a in *T. weissflogii*. In the other two species, however, fluorescence per cell was roughly proportional to chlorophyll a at low values of cellular chlorophyll a, but increased less steeply in highly pigmented cells. Trends in cellular content of the chlorophylls and carotenoids and in fluorescence excitation spectra indicate that changes in relative abundance of chlorophyll a and accessory pigments cannot explain the observed changes in fluorescence as a function of chlorophyll a per cell. Instead, results indicate that changes in the absorption cross-section, determined by absolute pigment content and cell size, dictate changes in fluorescence per chlorophyll a within a species. Evidence is also given supporting the direct comparability of results from different flow cytometry systems provided differences in excitation source are taken into account. Discussion includes a consideration of implications for field applications of flow cytometry related to inferences about the pigment content and light history of algal cells from their fluorescence properties.

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INTRODUCTION

PIGMENT AND FLUORESCENCE MEASUREMENTS: HISTORICAL PERSPECTIVE

The potential for pigment and fluorescence measurements to contribute significantly to our understanding of primary productivity in the sea is widely recognized by oceanographers. A variety of methods have been developed to identify, quantify and characterize the distribution and physiology of photosynthetic organisms. The development of techniques to measure chlorophyll (chl) a content in oceanic waters (Richards and Thompson, 1952) has been critical to progress in this area. These innovations led to a search for relationships between chl a concentration and parameters like photosynthetic biomass and productivity (Ryther and Yentsch, 1957; Yentsch, 1962; Strickland, 1965).

With the advent of fluorometric methods came the capability to rapidly and quantitatively monitor *in vivo* chl a fluorescence at sea and the characterization of photosynthetic biomass in the ocean became more rapid and routine than ever attainable by microscope enumeration (Lorenzen, 1965; Lorenzen, 1966; Anderson, 1969; Hobson and Lorenzen, 1972). Spectrofluorometry has led to the evaluation of the potential for fluorescence spectral differences to contribute to phytoplankton community characterization (Yentsch and Yentsch, 1979; Yentsch and Phinney, 1985) and suggestions that the ratio of fluorescence emission for different excitation wavelengths may predict such properties as photosynthetic and growth efficiency (Glover et al., 1987). At the same time, the development of techniques to sense absorption and fluorescence properties by aircraft and satellite (e.g. Yentsch and Yentsch, 1984;

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Prezelin and Boczar, 1986) has introduced the potential for water body characterization on scales previously unimaginable.

In recent years, the refinement of techniques to measure extracted pigment content has led to the growing use of chromatographic methods such as high performance liquid chromatography (HPLC) to separate and quantify the chlorophylls and carotenoids. This technology adds resolution to the analysis of community structure unattainable by *in vivo* fluorometric techniques. The presence of certain pigments indicates evidence of specific algal groups (Jeffrey, 1974;1980) whose distribution can thus be described at least qualitatively. Gieskes and Kraay (1986) have extended interpretation of HPLC data to propose pigment changes as evidence for physiological differences and Bidigare et al. (1986) correlated pigment distributions with physical processes to assess the importance of these processes in determining algal distributions.

None of the methods discussed thus far relate to the properties of individual cells. In each case quantitative measurements are bulk or average properties of all the cells in a sample. With the introduction of flow cytometry to oceanography comes the capability to measure optical properties of individual cells (Yentsch et al., 1983; Olson et al., 1985; Chisholm et al., 1988). Fluorescence and light scattering characteristics of individual cells can be measured rapidly and quantitatively and populations can be recognized by their characteristic flow cytometry "signatures" or groups of correlated parameters (light scatter and fluorescence of varied excitation and emission) (Olson et al., 1985; Olson et al., 1988). Single cell analysis allows for population boundaries to be assigned based on the distribution of the

appropriate optical parameters. Field work with flow cytometry is being expanded to include dual beam applications which allow crude excitation-emission spectra to be measured on individual cells. Olson et al. (1988) have used this technique to show that different pigment types of the cyanobacteria *Synechococcus* can be distinguished unambiguously in natural samples using flow cytometry. The result is a more detailed representation of the community structure than can be obtained with average pigment or fluorescence properties.

Work has also been done which exploits the ability of flow cytometry to distinguish cells belonging to the same species, but having different optical properties resulting from different growth histories. Olson et al. (1983) and Glibert et al. (1986) have documented fluorescence changes as a function of nitrogen availability and Cowles et al.(1988) exploited this to investigate whether copepod feeding is selective. Yentsch et al. (1985) has documented an increase in chl a fluorescence in a dinoflagellate grown at low light using flow cytometry and different responses of chl a content and flow cytometry chl a fluorescence have also been shown in two diatom species grown at various irradiances (Sakshaug et al., 1987).

Flow cytometric applications are currently limited by our knowledge of what the relevant optical parameters can tell us about the physiology of the cells. Interpretation of flow cytometry data, beyond description of population distributions, requires an understanding of how the measured parameters vary with cell conditions such as pigment content, size and physiological state. None of the studies discussed above have attempted to probe the physiological bases for flow cytometry fluorescence changes or to propose a mechanistic description of these

changes. Flow cytometric analysis correlated with bulk measurements such as pigment quantification can begin to answer these questions.

The goal of the present work is to describe in detail the relationship between flow cytometry fluorescence and pigment content in three species of eukaryotic marine phytoplankton grown over a range of irradiances and to discuss some of the basic considerations which contribute to this relationship with focus on implications for flow cytometry in field applications.

PIGMENT CHANGES IN RESPONSE TO CHANGING LIGHT

Pigment changes in eukaryotic algae adapted to different growth irradiances are well documented. Although responses vary widely, some generalizations can be made. The major light harvesting pigments, chlorophylls a,b and c and the carotenoids, fucoxanthin and peridinin, tend to be present in higher concentrations in cells grown at low light (Chan, 1978; Prezelin and Sweeney, 1978; Falkowski and Owens, 1980; Schlesinger and Shuter, 1981; Faust et al., 1982; Bates and Platt, 1984; Gallagher et al., 1984; Dubinsky et al., 1986; Sakshaug et al., 1987; Sukenik et al., 1988). In addition, the relative abundance of photosynthetic pigments often changes as a function of light adaptation. Commonly, the accessory pigment content increases relative to chl a as growth irradiance decreases, although the amount of response differs (Prezelin, 1976; Falkowski and Owens, 1980; Faust et al., 1982; Dubinsky et al., 1986; Glover et al., 1987; Sukenik et al., 1988). However, Perry et al. (1981) report an opposite trend in chl a : chl c for some species and pigment ratios have been found to be relatively constant for certain dinoflagellates (Faust et al., 1982; Dubinsky et al., 1986).

Even within three clones of the same diatom, responses of absolute pigment content and relative abundance of pigments to changes in growth light are different (Gallagher et al., 1984). More specifically, at the same light intensity, the absolute and relative amounts of pigments are different and changes induced by a shift in light intensity vary in magnitude between the clones.

While changes in specific pigments may be compared and interpreted, some caution must be taken when considering changes in total pigments or groups of pigments. For instance, reports of changes in total carotenoids are difficult to interpret in this context because some carotenoids are believed to play a photoprotective role in the photosynthetic apparatus (Larkum and Barrett, 1983; Prezelin and Boczar, 1986; Sieferman-Harms, 1987). Despite the generalization that accessory pigments often decrease in abundance with increasing light intensity, these pigments may be present at higher concentrations relative to chl a in high light cells, as observed for lutein in *Dunaliella tertiolecta* (Sukenik et al., 1988). Thus, increases in total carotenoids relative to chl a at high irradiance (Cook, 1963; Dubinsky et al., 1986) must be interpreted with caution since trends for specific carotenoids may be different.

MECHANISTIC FRAMEWORK FOR LIGHT ABSORPTION AND FLUORESCENCE

For the purpose of discussing the present work, a relatively simple view of the photosynthetic apparatus is appropriate. Within the cell, an area of protein bound light-harvesting pigment molecules and complexes surround the photochemical reaction center or trap chlorophyll (Fig. 1). The light harvesting pigments or antennae are composed of a
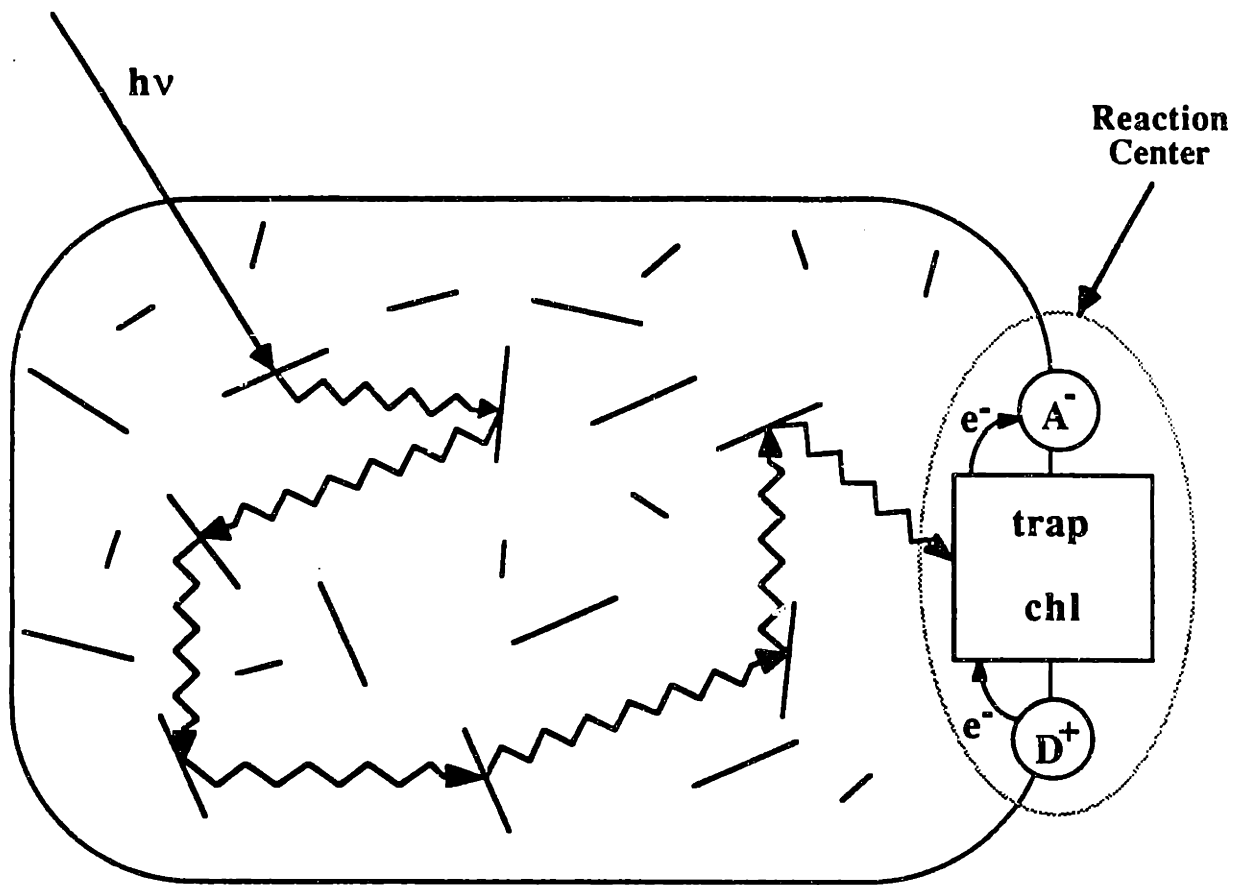
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Figure 1. Schematic of the light harvesting unit in a photosynthetic
cell where a photon (hν) is absorbed and transferred to the trap
chlorophyll. The acceptor (A) receives the excited electron from the
trap which is then reduced by a suitable electron donor (D). (Adapted
from Nobel, 1970).

variety of different pigments. These antennae molecules are capable of
harvesting incident photons with the probability of successful
absorption dependent on the wavelength of the incident light. Each type
of pigment molecule preferentially absorbs light of specified
wavelengths.

Absorption of a photon by a pigment molecule causes the excitation
of an electron above the ground state. There are essentially three
possible pathways for this exciton to follow. Through resonance-type
transfer the energy can migrate between the antennae molecules and make
its way to the reaction center (Fig. 1). Once the reaction center is
reached, the excited electron may be accepted by the photochemical
electron transport chain and ultimately contribute to carbon fixation.
A second possibility is that at any point in the transfer process, the
currently excited electron may fall back to the ground state releasing
the energy radiationlessly as heat. Finally, after transfer to chl a
has occurred, the excitation energy may be re-emitted as light of longer
wavelength, i.e. as fluorescence. It is important to distinguish that
photons can be absorbed by any of the light harvesting molecules but, *in
vivo*, fluorescence is only emitted by chl a (except in cells containing
phycobiliproteins). Although the mechanisms for energy transfer are
poorly understood, it has been observed that transfer efficiencies to
chl a approach 100% and fluorescence from the accessory pigments *in vivo*
is essentially undetectable (Karukstis and Sauer, 1983; Larkum and
Barrett, 1983; Sieferman-Harms, 1985; Prezelin and Boczar, 1986).

With this scheme for reference, it is apparent that fluorescence
emission intensity for a given excitation intensity can be considered to
depend first, on the ability of the cell to absorb photons and second,

on the set of factors which affect the fraction of absorbed photons which ultimately contribute to fluorescence. Any conditions that change the probability of an exciton being dissipated as heat or going to carbon fixation fall in the latter category. A clear example is the redox state of the primary acceptor in the photochemical electron transport chain. If the acceptor is not oxidized, the reaction center is effectively closed and more fluorescence will be emitted. As described by Sivak and Walker (1985), this mechanism is proposed to account for some of the characteristics of the fluorescence induction curve observed upon dark to light transition (Bates and Platt, 1984; Owens, 1986) and in the same manner can explain changes in the measured fluorescence yield (Falkowski et al., 1986).

In addition, other non-photochemical factors have been proposed to play a role in determining how much fluorescence will be emitted for a given amount of absorbed energy. A relationship has been observed between the proton gradient across the thylakoid membrane and fluorescence quenching. As the gradient increases, structural changes in the membrane are proposed to cause increased dissipation of energy as heat and thus reduce the fluorescence intensity (Sivak and Walker, 1985). Recently, it has been suggested that external factors such as light and temperature also influence the degree to which fluorescence is affected by non-photochemical quenching mechanisms (Laasch, 1988).

Factors which affect the ability of the cell to absorb incident photons are related to the abundance and distribution of the light harvesting pigments. Since the pigments preferentially absorb different wavelengths, changes in the relative abundance of the various pigments will alter the cells' absorbing efficiency at a particular wavelength

for a given incident spectrum of light (Kirk, 1983). At the same time, intracellular self-shading, which is also spectrally dependent, is a function of the absolute concentration of absorbing molecules. At sufficiently high concentrations, light is attenuated within the cell and photon absorption per molecule of pigment is decreased (Ramus, 1978; Kirk, 1986; Morel and Bricaud, 1986). This effect can also be dependent on the structure and distribution of chloroplasts within the cell (Kiefer, 1973). Compact chloroplasts represent locally higher pigment concentrations and an increased potential for self-shading.

*   *   *   *   *   *   *

In the work presented in this paper, three species, a diatom *Thalassiosira weissflogii*, a coccolithophorid *Hymenomonas carterae*, and a dinoflagellate *Amphidinium carteri* were grown over a range of light intensities which induced corresponding variations in cellular pigment content. Flow cytometry fluorescence per cell, bulk *in vivo* fluorescence, and fluorescence excitation spectra were measured on each culture and cellular chlorophylls and carotenoids were quantified using HPLC. Our goal was to describe and interpret flow cytometry fluorescence signals with respect to relative pigment content and cellular light absorption efficiency. This understanding allows us to put boundaries on the interpretation of fluorescence signals measured in the field and on inferences about the light history of cells made from their fluorescence properties.

METHODS

CULTURE CONDITIONS

Cultures of *Thalassiosira weissflogii*, *Hymenomonas carterae* and *Amphidinium carteri* were maintained in exponential growth in nutrient replete f/2 media (Guillard,1975) at 20°C. Constant illumination was supplied by Sylvania cool white fluorescent lamps at intensities ranging from 10 to 1000 $\mu Em^{-2}sec^{-1}$. Three cultures were maintained at each of 7 to 9 intensities for each species. The light level gradient was attained using neutral density plastic window screen and intensities were measured with a Biospherical Instruments, Inc. (San Diego, CA) QSL-100 Quantum Scalar Irradiance Meter. Growth rate was monitored by fluorescence with a Turner Designs Fluorometer Model 10 (Mountain View, CA).

SAMPLING

Adaptation to the various light intensities was considered complete when chlorophyll a per cell and red fluorescence per cell remained constant. Analysis was not conducted until at least ten generations had occurred in constant conditions. Measurements included cell counting and sizing on a Coulter Electronics (Hialehia, FL) Model Z_m/C256 electronic particle counter, bulk *in vivo* fluorescence, pigment analyses, flow cytometric analysis, and fluorescence excitation spectra. All fluorescence measurements were made after at least 15 minutes of dark incubation.

PIGMENT EXTRACTION AND ANALYSES

Samples were filtered onto Schleicher and Schuell #34 glass fiber filters and extracted for 48 hours in 90% acetone under refrigeration. After homogenization with a Teflon tissue grinder, chlorophyll a concentration was determined fluorometrically (Parsons et al., 1984). The method was calibrated spectrophotometrically using the equations of Jeffrey and Humphrey (1975).

Additional aliquots collected on glass fiber filters were frozen rapidly in liquid nitrogen for subsequent HPLC analysis. One sample from each light intensity for *T. weissflogii* and *H. carterae* and two from each for *A. carteri* were extracted and ground in 100% acetone and analyzed for chlorophyll and carotenoids by reverse-phase HPLC (Mantoura and Llewellyn, 1983).

FLUORESCENCE MEASUREMENTS

The bulk *in vivo* fluorescence of cell suspensions was determined using a Turner Designs Fluorometer Model 10 (Mountain View, CA). Excitation was between 400 and 460 nm and red emission was measured at greater than 640 nm. Fluorescence values were normalized by cell density for each culture.

Quantum corrected fluorescence excitation spectra were measured on suspensions of cells using an SLM Aminco (Champagne-Urbana, IL) SPF-500C spectrofluorometer. Within a species, cell concentration was adjusted to give the same optical density at 440 nm for each culture. The excitation source was a xenon arc-lamp and excitation wavelength was varied in 1 nm increments from 360 to 600 nm with a 2 nm slit width.

Emission was monitored at 680 nm with a 20 nm width. Spectra were stored by computer for later smoothing and analysis. A blank run with culture medium was subtracted from each spectra.

Flow Cytometry

Fluorescence measurements were collected for thousands of cells from each culture using each of two flow cytometers (see below). Data was collected using log amplifiers and stored by computer. As discussed in Olson et al. (1988) fluorescent beads were used as a standard. Before analysis, fluorescent beads were added to each cell suspension. All data was linearized and the mean fluorescence for the bead and cell distributions was computed. All relative fluorescence data is expressed on a per cell basis and represents the mean fluorescence value for the cell distribution divided by the mean value for the bead distribution. This normalization allows the accurate comparison of relative fluorescence between different cultures since the beads act as a standard.

All samples were first analyzed using a microscope based flow cytometer or "Cytomutt" (Shapiro, 1985), described by Olson et al. (1983). Excitation illumination consisted of the blue lines of a mercury arc lamp where the majority of the interrogation intensity is at 436 nm. Fluorescence was measured as emitted light passing a 600 nm long pass filter. The standards used were 2.1 μm diamter Nile Red beads (Pandex; Mundelein, IL) for *T. weissflogii* and 2.24 μm diameter Fluoresbrite beads (Polysciences,Inc; Warrington, PA) for *H. carterae* and *A. carteri*.

All samples were also analyzed on a Coulter EPICS V flow cytometer (Coulter Electronics; Hialeah, FL) modified as described in Olson et. al. (1988). The excitation source was an argon ion laser running in all lines mode with a total output of 500 mW. Each cell was excited first with the 488 nm line and after approximately 7 μs with the 515 nm line. The details of this configuration are given in Olson et al. (1988). Fluorescence monitored consisted of emitted light passing a 680 nm bandpass filter (±20 nm bandwidth). Forward light scatter (FLS) was also collected and this data, as well as fluorescence for 515 nm excitation, are given in Appendix A. The standard was Nile Red beads for all samples.

The results from flow cytometric analysis on the Cytomutt will first be presented for each of the species. Following this section, the results from the two flow cytometry systems will be directly compared. The general conclusions of the thesis are not affected by which set of flow cytometry data is considered.

SPECIES COMPARISON

Thalassiosira weissflogii

Consistent with results previously reported (Dubinsky et al., 1986; Falkowski et al., 1985; Post et al., 1984), in *T. weissflogii* chl a per cell increased three-fold as growth rate decreased due to light intensites varying between 10 and 1000 μE m^{-2} sec^{-1} (Fig. 2A). Cell volume decreased as a function of light intensity, however, chl a per volume was somewhat higher in the low light cells (Fig. 2B) due to the dramatic increase in the amount of chl a in these cells. The pigment increase at low irradiance resulted in a more than two-fold change in the fluorescence per cell measured by flow cytometry. Fluorescence was related linearly to chl a per cell with an extrapolated positive fluorescence value at zero chl a per cell (Fig. 3A). A similar trend was observed for bulk *in vivo* fluorescence normalized by cell density (Fig. 3B).

Fluorescence excitation spectra on whole cells showed consistently three main peaks in fluorescence emission measured at 680 nm (Fig. 4). These correspond to excitation at approximately 440 nm, 490 nm and 535 nm and are indicative of absorption by chl a, chl c and fucoxanthin, respectively (Jeffrey, 1980; Prezelin and Boczar, 1986). The relative magnitude of these peaks changed markedly as a function of growth light intensity (Fig. 4). The fluorescence intensity was similar for excitation at the three peak wavelengths when cells were grown at low light. As the light intensity for growth increased, however, the peaks in emission due to excitation at 490 nm and 535 nm decreased relative to

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Figure 2. Growth rate, cell volume and chl a as a function of growth
light intensity in the diatom T. *weissflogii*. A) Growth rate and chl a
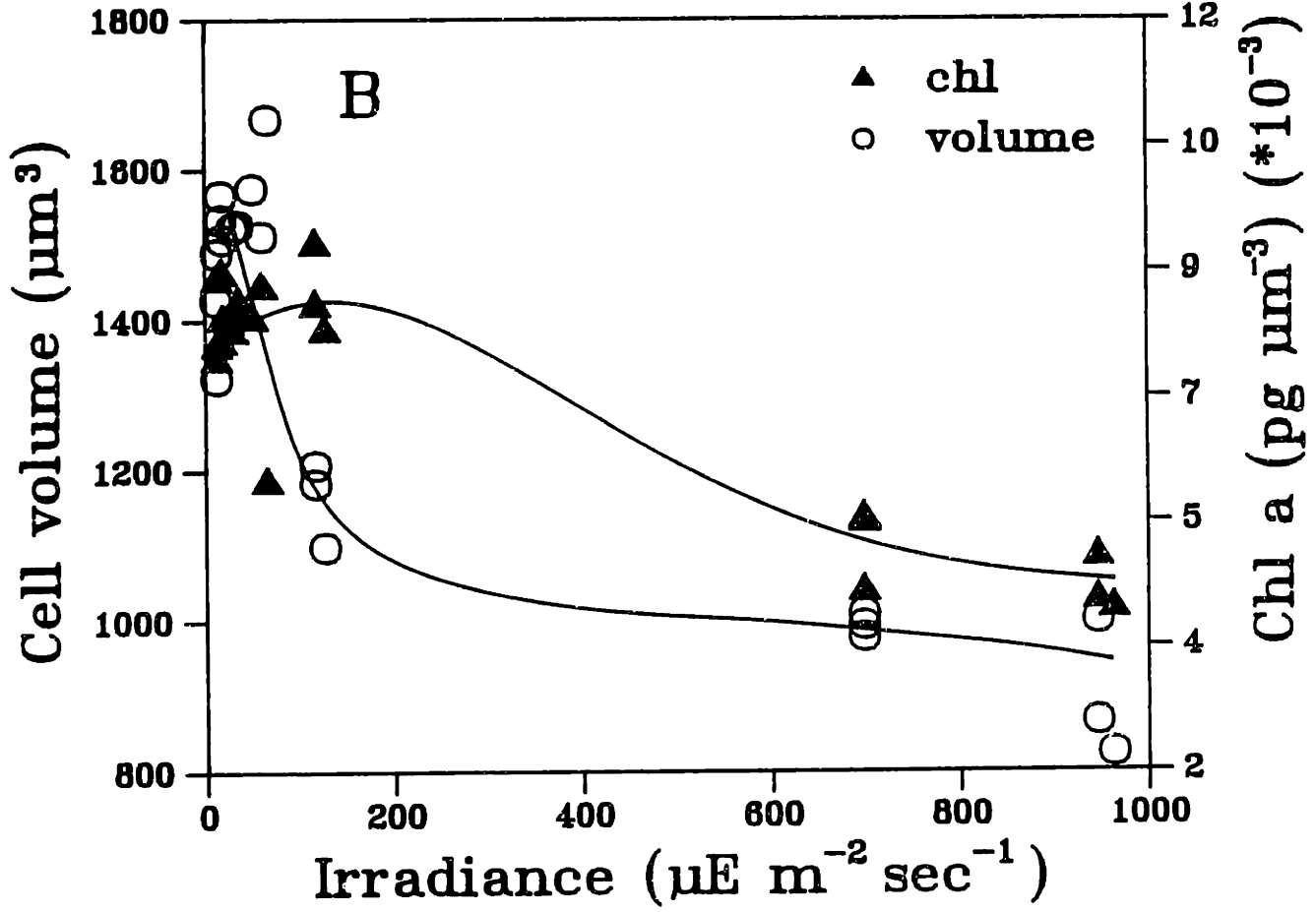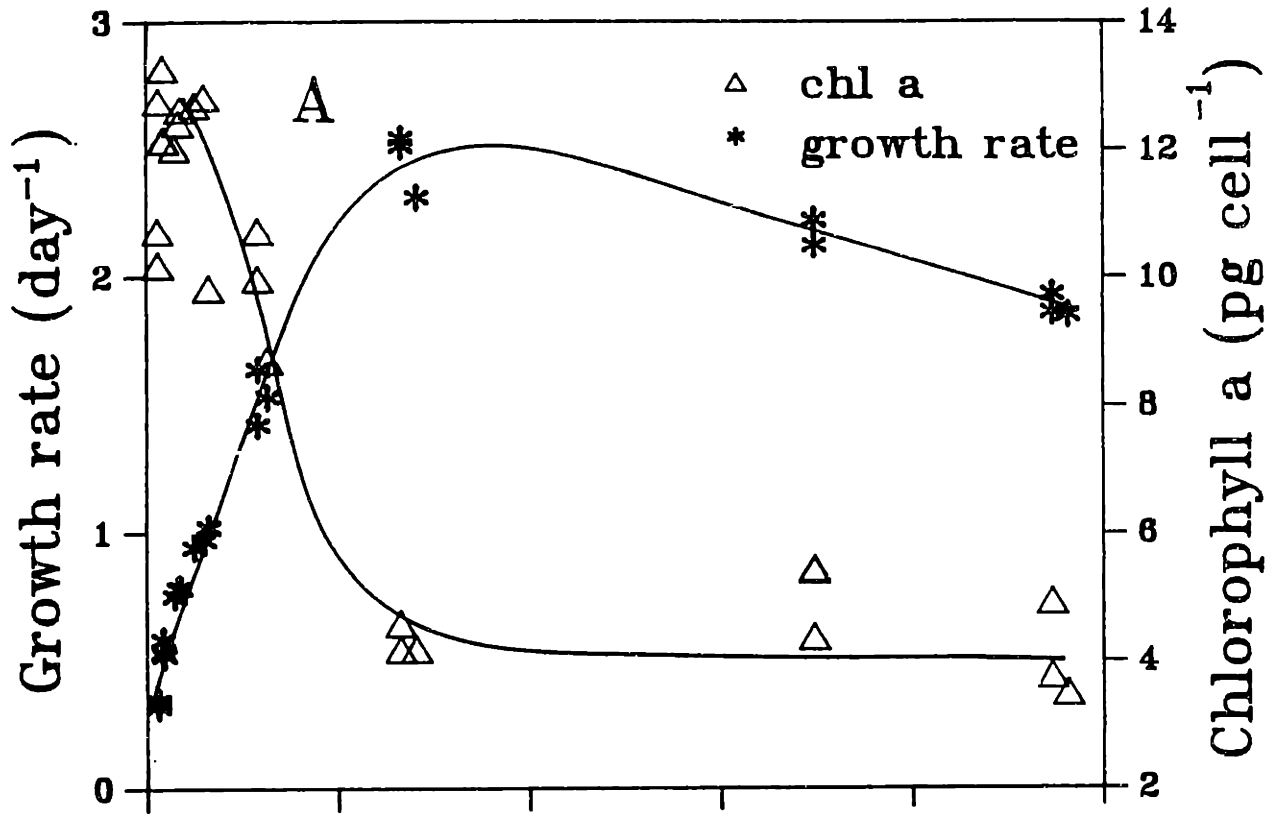per cell. B) Cell volume and chl a per volume.

Figure 3. Red fluorescence per cell as a function of chl a per cell in
T. weissflogii. A) Fluorescence measured on individual cells using the
Cytomutt flow cytometer. B) Bulk *in vivo* fluorescence measured in a
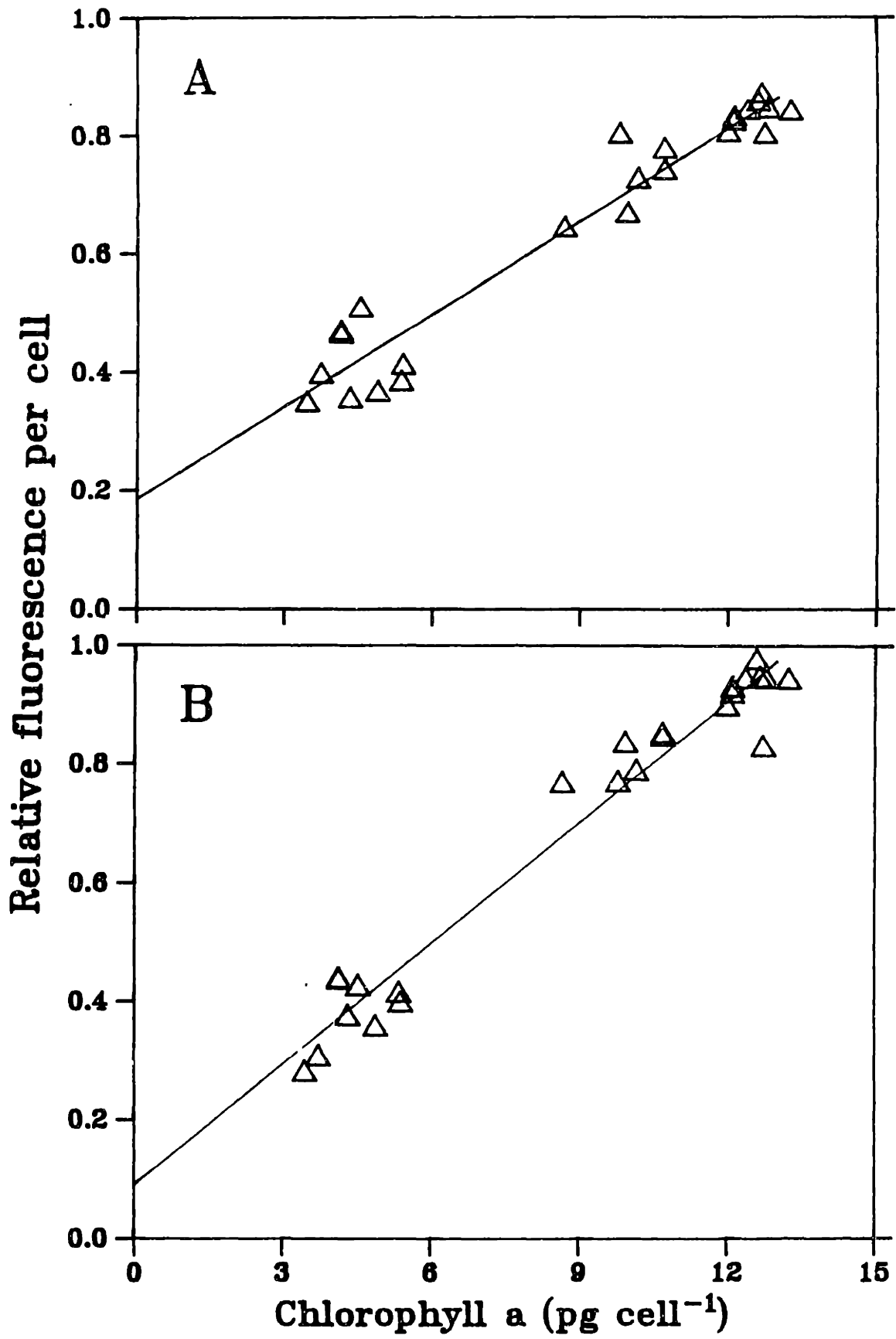fluorometer and normalized by cell concentration.

Figure 4. Representative fluorescence excitation spectra on suspensions of whole cells of *T. weissflogii* grown over a range of irradiances. Fluorescence emission was measured at 680 nm.
A) Low light: 30 $\mu Em^{-2}sec^{-1}$. B) Medium light: 120 $\mu Em^{-2}sec^{-1}$.
C) High light: 950 $\mu Em^{-2}sec^{-1}$. Relative scale is different for each sample.

Relative fluorescence (y-axis)

Excitation wavelength (x-axis)

A Low

B Medium
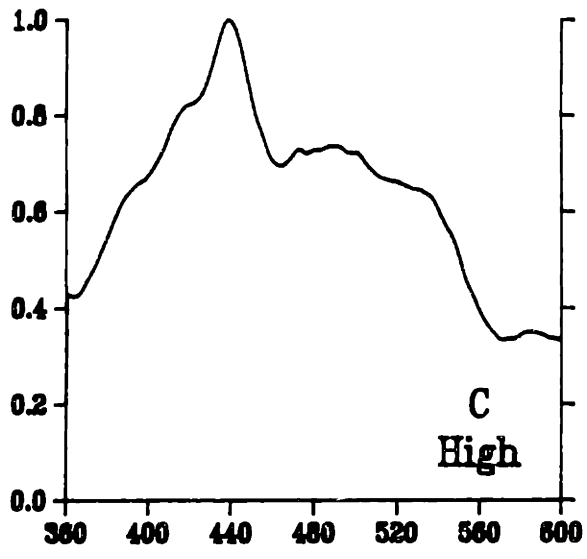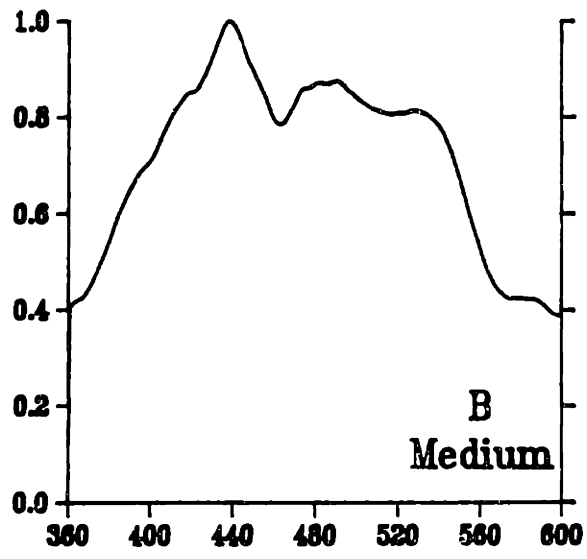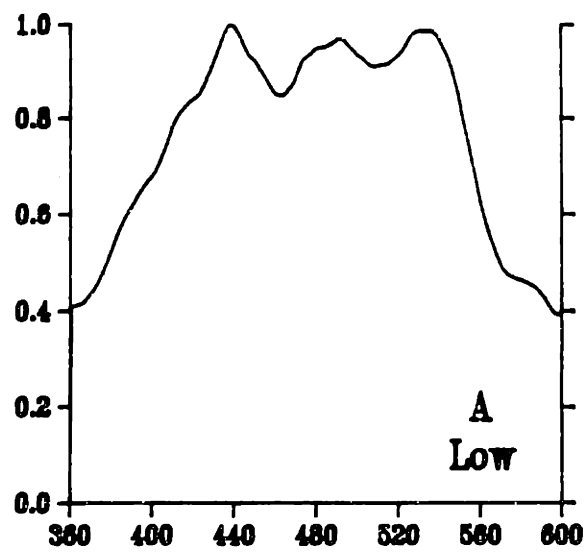
C High

excitation at 440 nm.

HPLC analysis of cell extracts showed that chl a, chl c and the carotenoid fucoxanthin decreased on a cellular basis as growth irradiance increased at sub-saturating light intensities (Fig. 5). As noted in work with other species (Chan, 1978; Falkowski, 1980; Prezelin and Sweeney, 1976), these pigments did not continue to increase in cells grown at the lowest photon flux densities; nor did they change over a range of saturating light intensities. Concentrations of chl a varied from about 3.5 to 12 pg cell^{-1}; fucoxanthin ranged from 1 to 4 pg cell^{-1} and chl c from 0.25 to 1.0 pg cell^{-1}. The molar ratio of chl a to chl c was near 7 for cells under growth limiting light conditions and rose to 10 after light became saturating; chl a to fucoxanthin was nearly constant at a molar ratio just over 2 regardless of growth light intensity (see Discussion for details). Carotenoids other than fucoxanthin (β-carotene, and the epoxide cycle carotenoids, diadinoxanthin and diatoxanthin) were also quantified (see Appendix A) Both the chl a to β-carotene and the diadinoxanthin to diatoxanthin ratios decreased at high light consistent with photoprotective functions for β-carotene (Sieferman-Harms, 1987) and the epoxide cycle carotenoids (Prezelin and Boczar, 1986; Larkum and Barrett, 1983).

Hymenomonas carterae

Trends of growth rate and chl a per cell as a function of light intensity were similar in the coccolithophorid *H. carterae* (Fig. 6A). Growth saturated at approximately the same light level as *T. weissflogii* although at a lower growth rate: 1.4 day^{-1} versus 2.5 day^{-1} for the diatom. Also in contrast to *T. weissflogii*, there was no inhibition of
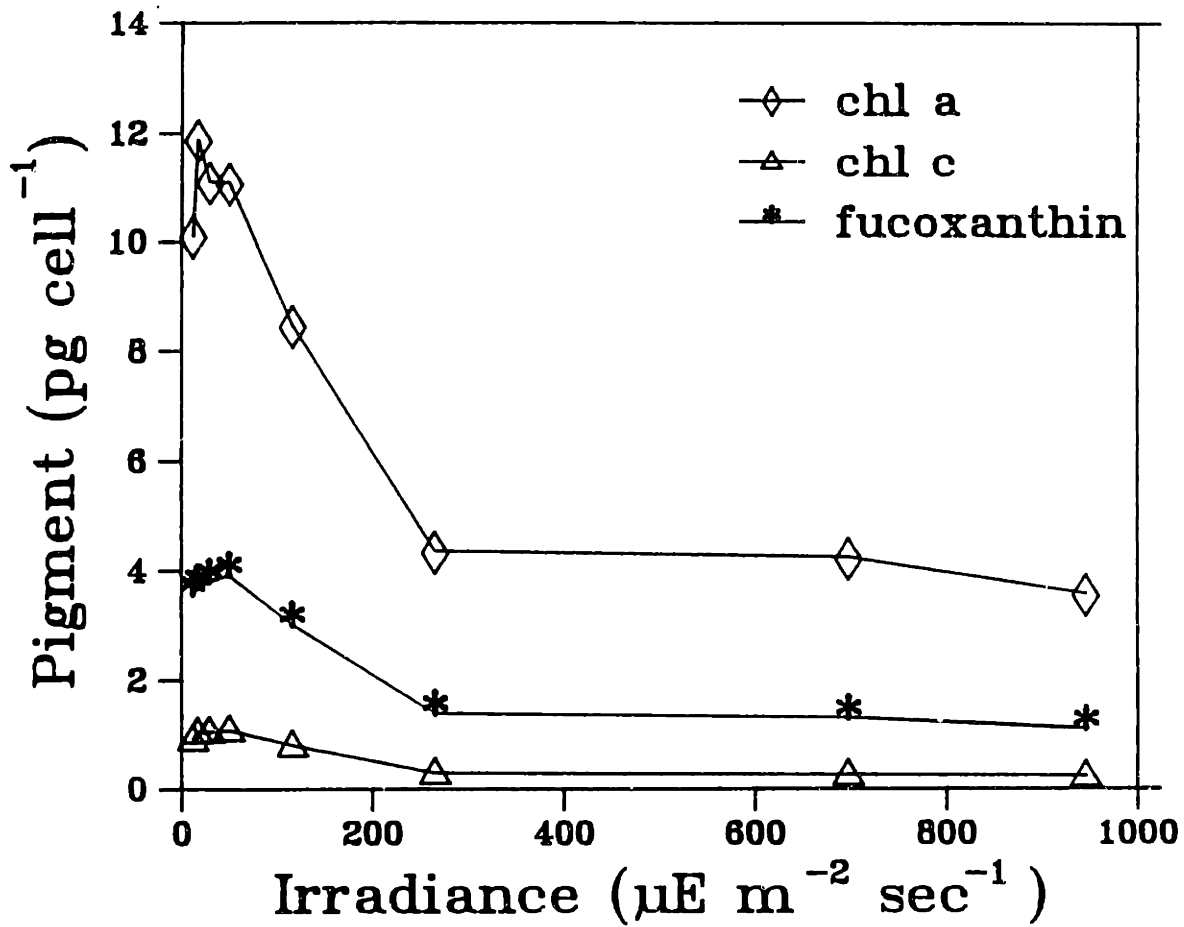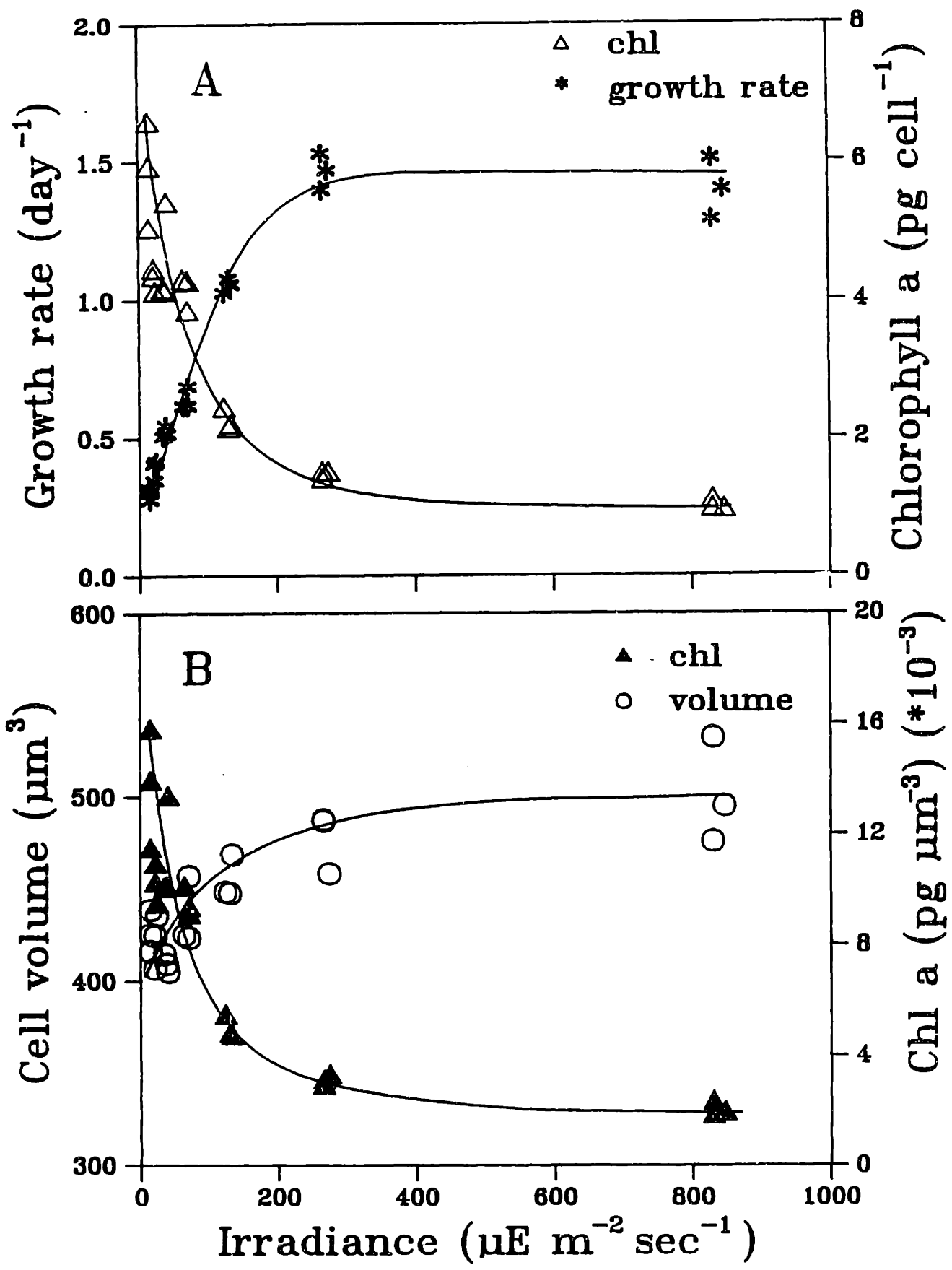
Figure 5. Cellular chl a, chl c and fucoxanthin quantified by HPLC in
T. weissflogii grown at different light intensities.

Figure 6. Growth rate, cell volume and chl **a** as a function of growth light intensity in the coccolithophorid *H. carterae*. A) Growth rate and chl **a** per cell. B) Cell volume and chl **a** per volume.

growth rate at high photon flux densities and cell volume increased with light intensity, contributing to a wider range of chl a per volume (Fig. 6B). Despite similar values of chl a per volume, under all conditions the chl a per cell was lower for *H. carterae* than observed for *T. weissflogii*. Correspondingly, bulk *in vivo* fluorescence per cell and average fluorescence per cell measured by flow cytometry were consistently lower for the coccolithophorid. While each of these fluorescence measurements was linear with chl a per cell for *T. weissflogii*, the relationship is less straightforward for *H. carterae* (Fig. 7). For chl a values less than about 3 pg cell^{-1}, relative fluorescence per cell increased linearly. However, as chl a continued to increase, the slope of the curve decreased markedly. This result was consistent for bulk *in vivo* fluorescence measurements and for flow cytometric analysis (Fig. 7).

In vivo fluorescence excitation spectra with emission monitored at 680 nm show three broad peaks at approximately 440 nm, 470 nm and 530 nm (Fig. 8). As for the diatom, these peaks are indicative of absorption by chl a, chl c and fucoxanthin. Also consistent with the results for *T. weissflogii*, the relative peak heights shift dramatically for cultures at different light intensities (Fig. 8). High light cells showed relatively less fluorescence due to excitation at the longer wavelengths compared with low light cells.

As expected, chl a, chl c and fucoxanthin per cell decreased in cells grown in high light (Fig. 9). Chl a ranged from less than 1 to nearly 6 pg cell^{-1}; chl c and fucoxanthin varied by ten-fold. The molar ratio of chl a to chl c was 7-8 for all light intensities except the highest where it increased to nearly 12, while chl a to fucoxanthin

Figure 7. Red fluorescence per cell as a function of chl a per cell in *H. carterae*. A) Fluorescence measured on individual cells with the Cytomutt flow cytometer. B) Bulk *in vivo* fluorescence measured in a fluorometer and normalized by cell concentration.

A

B

Relative fluorescence per cell
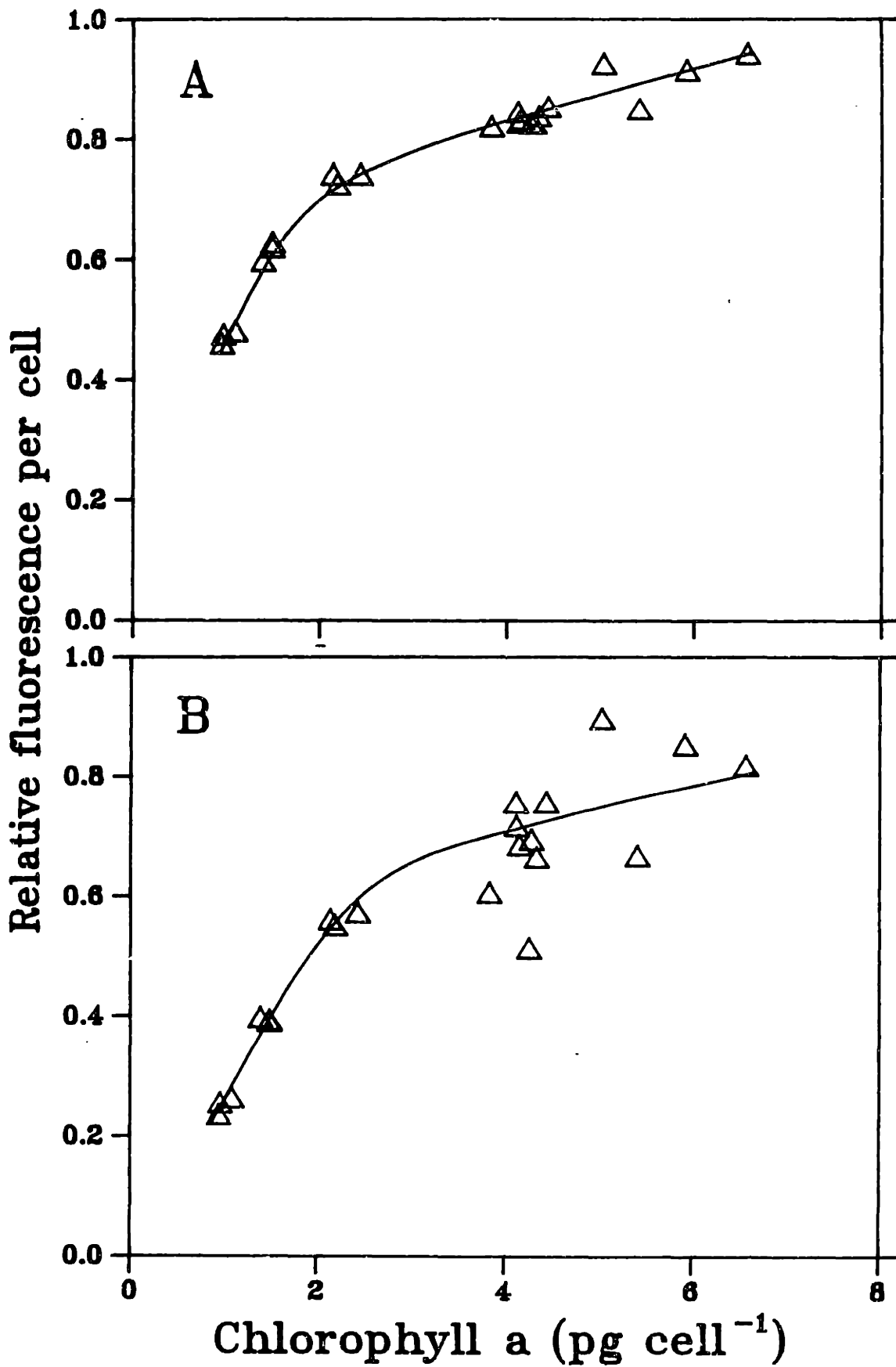
Chlorophyll a (pg cell^{-1})

Figure 8. Representative fluorescence excitation spectra on suspensions of whole cells of *H. carterae* grown over a range of irradiances. Fluorescence emission was measured at 680 nm.
A) Low light: 20 $\mu Em^{-2}sec^{-1}$. B) Medium light: 120 $\mu Em^{-2}sec^{-1}$.
C) High light: 850 $\mu Em^{-2}sec^{-1}$. Relative scale is different for each sample.

Relative fluorescence (y-axis)

Excitation wavelength (x-axis)
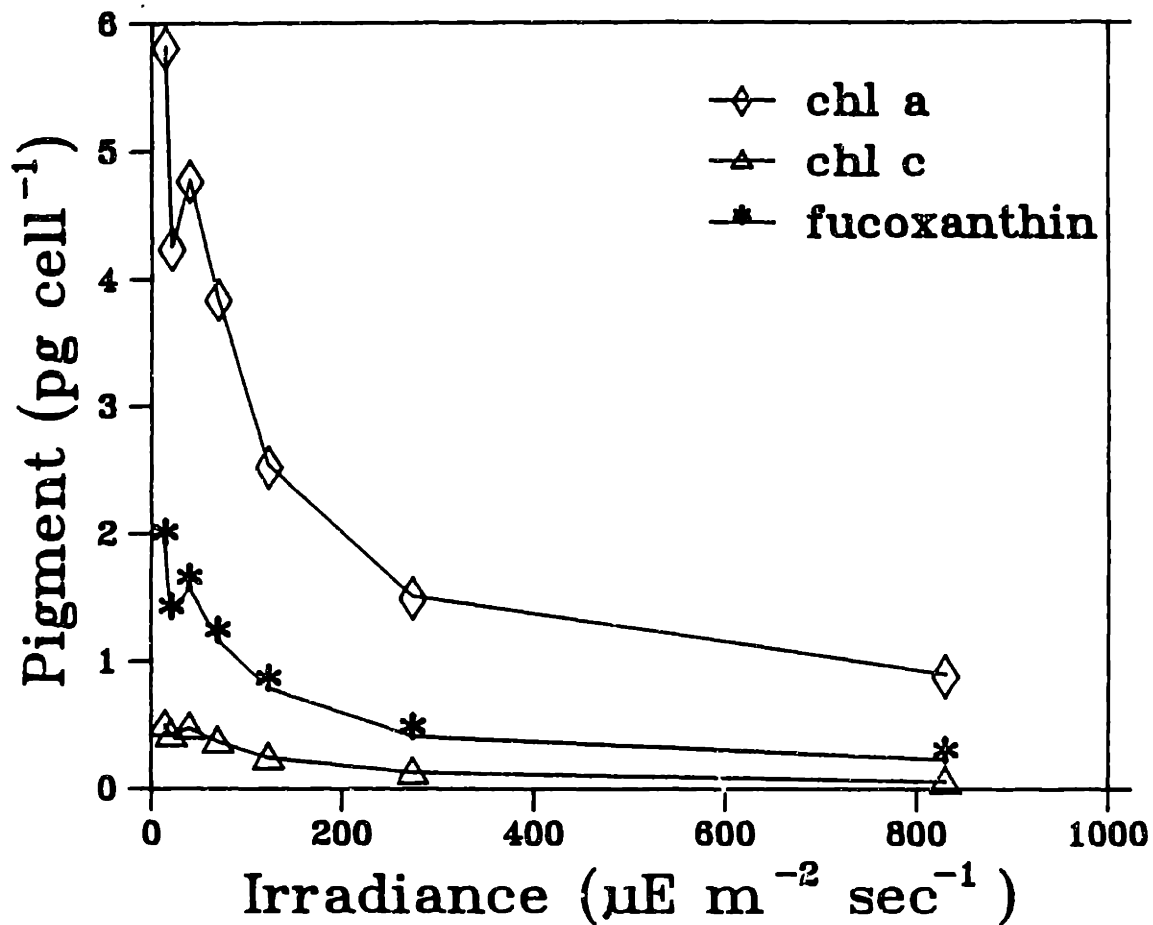
A
Low

B
Medium
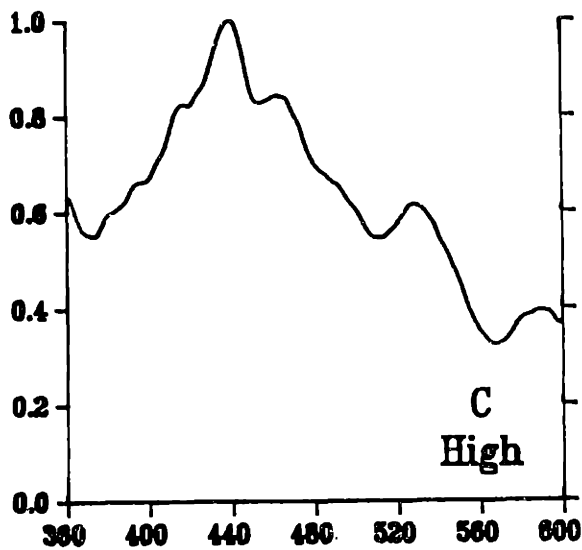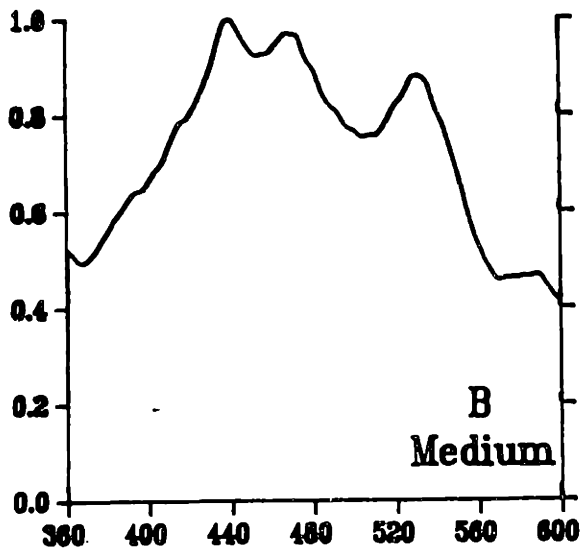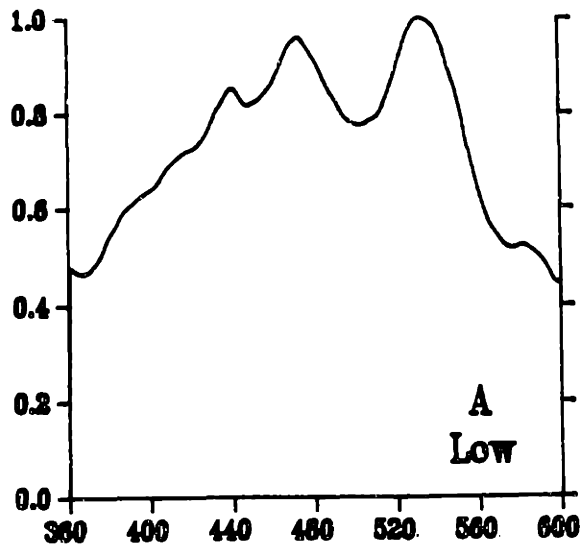
C
High

Figure 9. Cellular chl a, chl c and fucoxanthin quantified by HPLC in *H. carterae* grown at different light intensities.

varied from 2.1 at the lowest irradiance to 2.9 at the highest (see Discussion for details). Also detected were β-carotene, diadinoxanthin and traces of diatoxanthin (see Appendix A).

Amphidinium carteri

Growth rate of the dinoflagellate *A. carteri* saturated at less than 100 $\mu Em^{-2}sec^{-1}$ and the major increases in chl a per cell occurred at less than 200 $\mu Em^{-2}sec^{-1}$ (Fig. 10A). This low light saturation for dinoflagellates compared to other species has been shown previously (Chan, 1978). As in *H. carterae*, cell volume was generally lower in low light cells and the range of chl a per volume was relatively broad as compared to *T. weissflogii* (Fig. 10B). Also consistent with results in *H. carterae*, the relationship between fluorescence and chl a was not linear (Fig. 11). At chl a concentrations of more than 2 pg cell^{-1}, fluorescence per cell changed only slightly. As in both of the other species, flow cytometry and bulk fluorescence measurements yielded the same trends (Fig. 11).

Fluorescence excitation spectra for the dinoflagellate as a function of growth irradiance, showed similar trends as observed for the previous two species. As growth light intensity decreased, the contribution to fluorescence from absorption by the accessory pigments increased relative to chl a (Fig. 12). The peaks in fluorescence emission occur at excitation wavelengths of approximately 440 nm, 475 nm and 530 nm. In contrast to results for *T. weissflogii* and *H. carterae*, the 440 nm chl a peak appears generally as a shoulder and the chl c peak dominates the spectrum. This can be explained by the relatively higher chl c to chl a content of *A. carteri* and the stronger absorption in the

Figure 10. Growth rate, cell volume and chl a as a function of growth
light intensity in the dinoflagellate *A. carteri*. A) Growth rate and
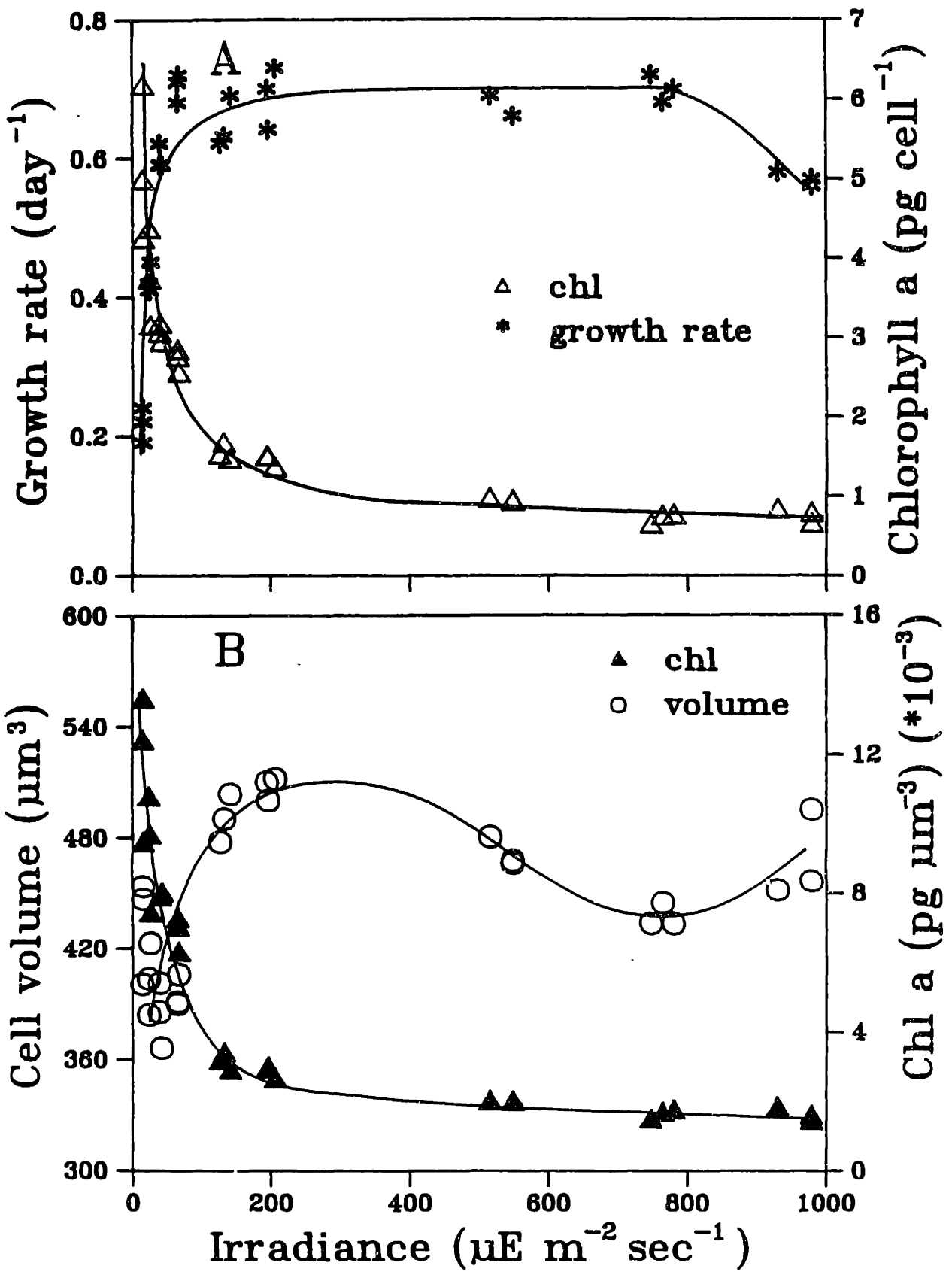chl a per cell. B) Cell volume and chl a per volume.

Figure 11. Red fluorescence per cell as a function of chl a per cell in
A. carteri. A) Fluorescence measured on individual cells using the
Cytomutt flow cytometer. B) Bulk *in vivo* fluorescence measured in a
fluorometer and normalized by cell concentration.
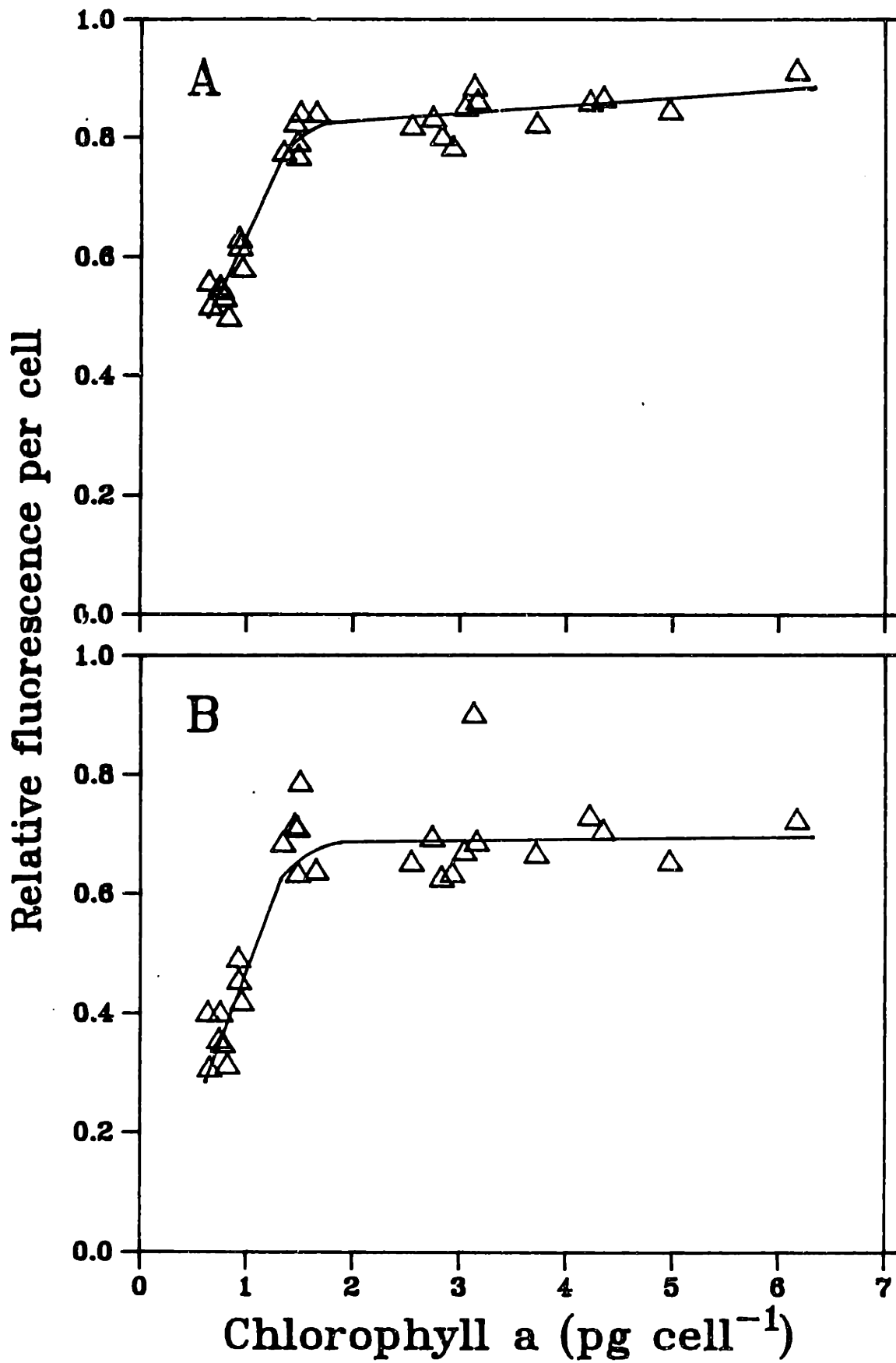
Figure 12. Representative fluorescence excitation spectra on suspensions of whole cells of *A. carteri* grown over a range of irradiances. Fluorescence emission was measured at 680 nm. A) Low light: 20 μEm^{-2}sec^{-1}. B) Medium light: 200 μEm^{-2}sec^{-1}. C) High light: 930 μEm^{-2}sec^{-1}. Relative scale is different for each sample.

Relative fluorescence

A
Low

B
Medium

C
High

Excitation wavelength

Soret region by chl c as compared to chl a (Jeffrey, 1980). The 530 nm peak in the excitation spectrum corresponds to absorption by the carotenoid peridinin.

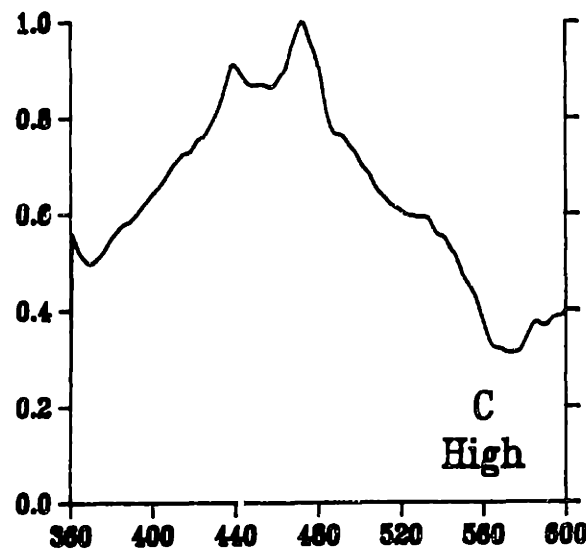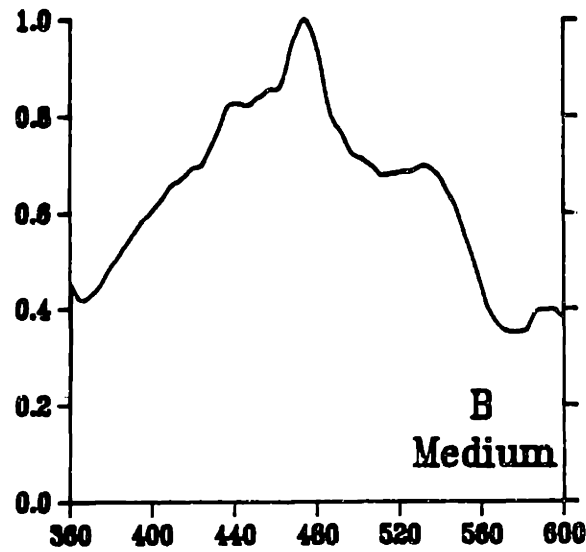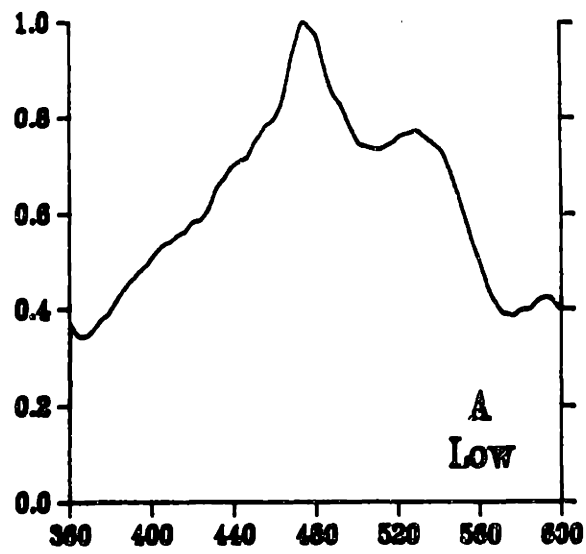HPLC pigment analysis revealed increases in the chlorophylls and peridinin at lower growth irradiances (Fig. 13). Chl a ranged from about 5 pg cell^{-1} at the lowest photon flux to just under 1 pg cell^{-1} at the highest; For the same light intensities, chl c ranged from about 1.5 to 0.2 pg cell^{-1} and peridinin from 3 to 0.5 pg cell^{-1} (Fig. 13). In addition, diadinoxanthin, diatoxanthin, β-carotene, and dinoxanthin were also quantified (see Appendix A). Molar ratios of chl a to chl c were 2.1-2.3 for irradiances of 200 μEm^{-2}sec^{-1} or less and increased to just over 3 at the highest light level; the chl a to peridinin ratio was consistently about 1.4 (see **Discussion** for details). As observed for T. *weissflogii*, increases were seen in β-carotene relative to chl a and in diatoxanthin relative to diadinoxanthin at high irradiances again consistent with the concept of a photoprotective role for β-carotene and the conversion of diatoxanthin to diadinoxanthin.

INSTRUMENT COMPARISON: CYTOMUTT VS COULTER EPICS V

Two types of flow cytometers are currently being used for the analysis of phytoplankton in the sea. The Coulter EPICS V, which is based on an argon ion laser (Olson et al, 1985, 1988) and the Becton Dickinson (Mountainview, CA) FACS Analyser, which uses a mercury arc-lamp (Li and Wood, in review) as is true of the Cytomutt system. To date, the fluorescence signals from these two types of systems have not been systematically intercalibrated. The present study represents an excellent opportunity for such a comparison.
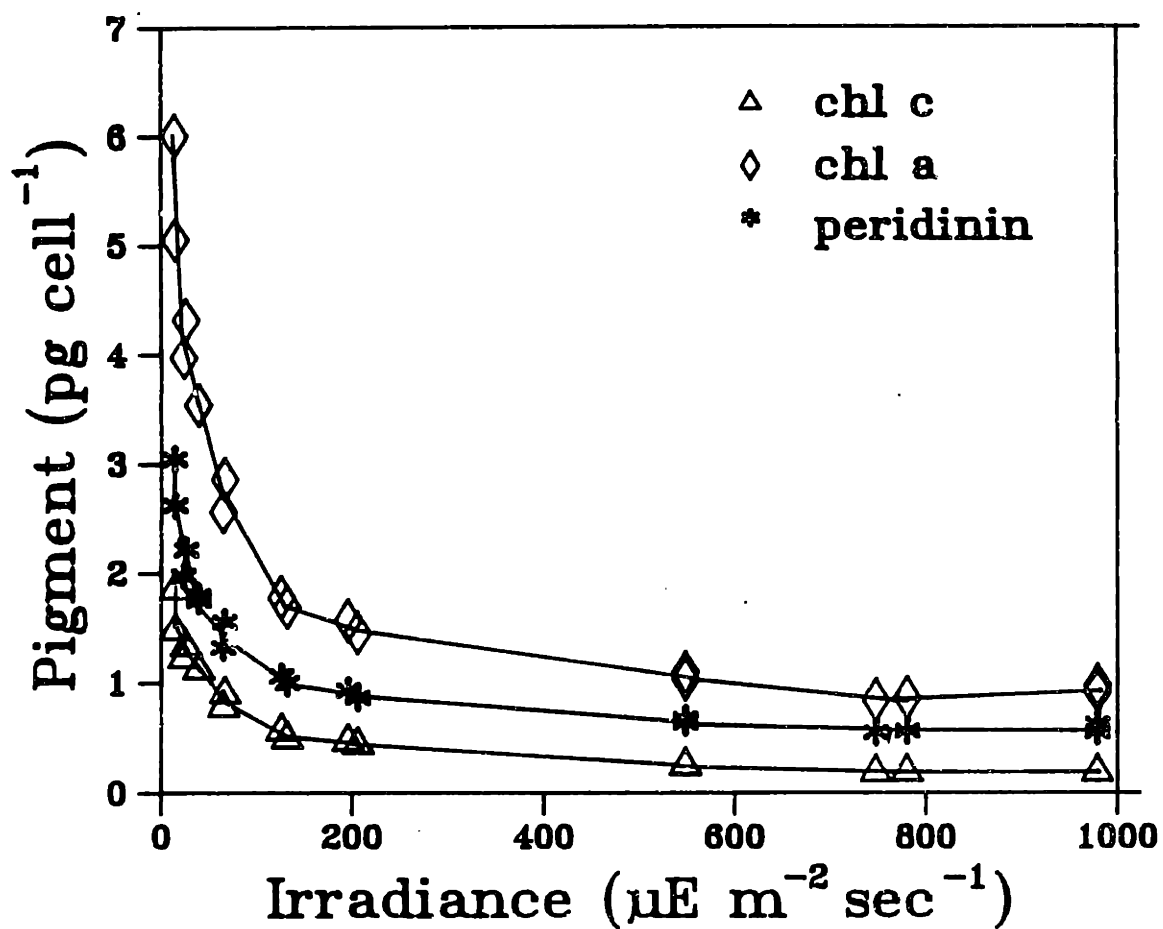
Figure 13. Cellular chl **a**, chl c and fucoxanthin quantified by HPLC in
A. *carteri* grown at different light intensities.

Although the results presented above were for flow cytometry analysis only on the Cytomutt, each culture from different light intensities for all three species was analyzed on two different flow cytometer systems as detailed in the Methods section. Recall that the excitation source for the Cytomutt was the blue lines of a Hg-arc lamp (mostly 436 nm) and for the EPICS was a 488 nm laser line. Although the trends between fluorescence and chl a per cell were generally similar for measurements on each system, there were some small but significant differences. For *T. weissflogii*, Cytomutt fluorescence per cell is directly proportional to similar EPICS fluorescence, despite the difference in excitation source (Fig. 14A). Measurements of *H. carterae* on the two systems also varied linearly; but, extrapolation indicates a zero signal on the EPICS corresponding to a positive signal on the Hg arc-based Cytomutt (Fig. 14B). The comparison is even more complex for *A. carteri*. For highly pigmented cells, the Cytomutt signal did not increase as rapidly as the EPICS signal (Fig. 14C). This is evident in a decrease in the slope of Cytomutt versus EPICS fluorescence per cell as fluorescence intensity increases.

This apparent discrepancy between the measurements on the two flow cytometers could be explained in large part by the changes in the fluorescence excitation spectra of the cells as a function of growth irradiance (Figs. 4, 8, and 12). From the fluorescence excitation spectra the ratio of emission when excited by 436 nm to emission when excited by 488 nm (Ex 436/488) was calculated and the flow cytometry fluorescence signals were corrected to account for changes in this ratio. Comparison of Cytomutt fluorescence per cell with EPICS fluorescence per cell multiplied by the ratio (Ex 436/488), for each

Figure 14. Comparison of fluorescence signals measured using the EPICS
with signals from the Cytomutt for cultures grown over a range of light
intensities. The same data is also shown after adjustment for
differences in excitation source between the two instruments (see text).
Fit curves are polynomials (third order in (A) and second order in (C)).

A T. weissflogii

B H. carterae

C A. carteri

adjusted data
raw data

EPICS fluorescence per cell
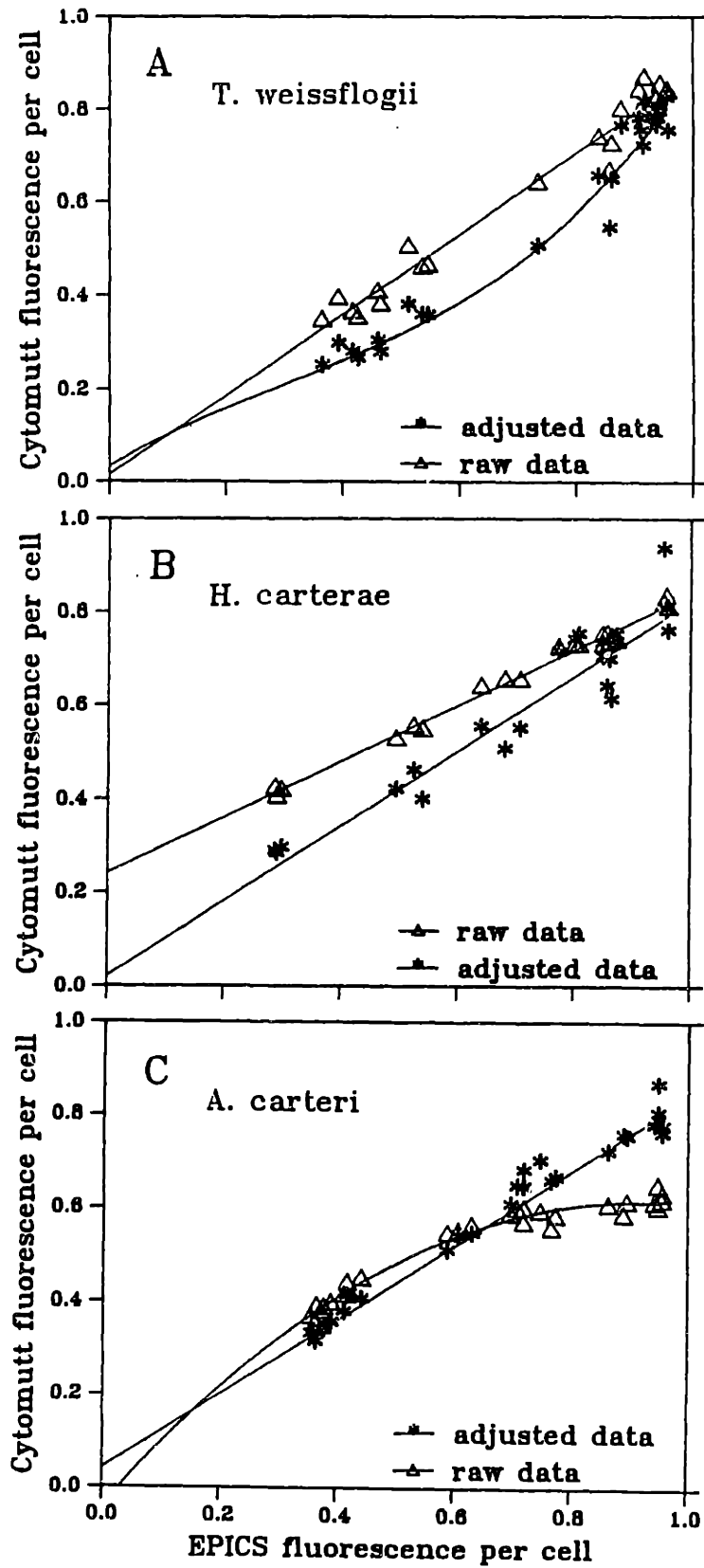
culture, shows direct proportionality for *H. carterae* and *A. carteri* (Figs. 14B,C). The same analysis for *T. weissflogii* shows only a small deviation from the original proportionality (Fig. 14A).

These results strongly suggest that the most significant difference between the Hg-arc lamp system and the argon ion laser system is the excitation source. There is also support for the direct comparability of relative flow cytometry measurements with spectrofluorometer measurements despite differences between the two methods in time scale of analysis and source intensity and bulk versus single cell analysis.

*   *   *   *   *   *   *   *

The complex and varied responses of these three species to different growth irradiance which are evident in the measurements reported in this paper pose questions as to the physiological and mechanistic relationship between cellular pigment composition and fluorescence per cell. Issues that must be addressed include the effects of changes in relative pigment abundance and electron transfer efficiency. In addition, light absorption efficiency as it is affected by absolute abundance of absorbing pigments and cell size must be considered. In the following section, the theoretical framework for this latter topic is reviewed.

THEORETICAL ANALYSIS

Beginning with the pioneering work of Duysens (1956), progress has been made toward the understanding of the absorption properties of individual algal cells (Kirk, 1975a,b; Kirk, 1976; Collins et al., 1985; Morel and Bricaud, 1986). Of particular relevance to the work presented here is the approach of Morel and Bricaud (1986) for determining the absorption efficiency of an individual cell.

ABSORPTION EFFICIENCY

Absorption efficiency (also referred to as absorptance) is defined as the ratio of the number of photons absorbed by a cell to the number of photons incident on the geometrical cross-section of the cell. This can also be interpreted as the ratio of absorption cross-section (A_a) to geometrical cross-section (s). With the assumption that the cell is spherical, homogeneous (i.e. evenly pigmented) and that its index of refraction is close to that of water, Beer's law for light absorption can be integrated to obtain:

$$Q_a = 1 + \frac{2(\exp^{-\rho})}{\rho} + \frac{2(\exp^{-\rho} - 1)}{\rho^2} \qquad (1)$$

which is valid at a single wavelength and expresses absorption efficiency, Q_a, as a function of the dimensionless parameter ρ, defined as:

$$\rho = a_s * d \qquad\qquad (2)$$

where d is the cellular diameter and a_s is the absorption coefficient of the cell material (Morel and Bricaud, 1986). The parameter, ρ, represents the optical thickness for absorption along a ray traversing the diameter of the cell. If a_s is expressed as the product of a specific absorption coefficient, a_i^* and an intracellular concentration, c_i:

$$a_s = a_i^* * c_i \qquad\qquad (3)$$

it is clear that ρ and thus Q_a depend on the size and pigment concentration of the cell.

For cells with relatively low values of the product $d * c_i$, Q_a varies in a linear fashion with ρ. However, at higher $d * c_i$, the absorption efficiency approaches saturation at $Q_a = 1$ (Fig. 15). This limit represents complete absorption by a black body which, by definition, is independent of increases in the optical thickness, ρ.

Morel and Bricaud (1986) also describe the derivation of an expression for Q_a in terms of an absorption coefficient for a suspension of identical spherical cells. This derivation relies only on the chlorophyll specific absorption coefficient, the intracellular chl a concentration and cell diameter. It is important to distinguish between the coefficient used to model absorption by a single cell and the coefficient for a suspension of these cells. Absorption by a homogeneous solution is different from absorption by a suspension of discrete absorbing packages. In general the specific absorption coefficient is higher for a homogeneous solution than for a suspension
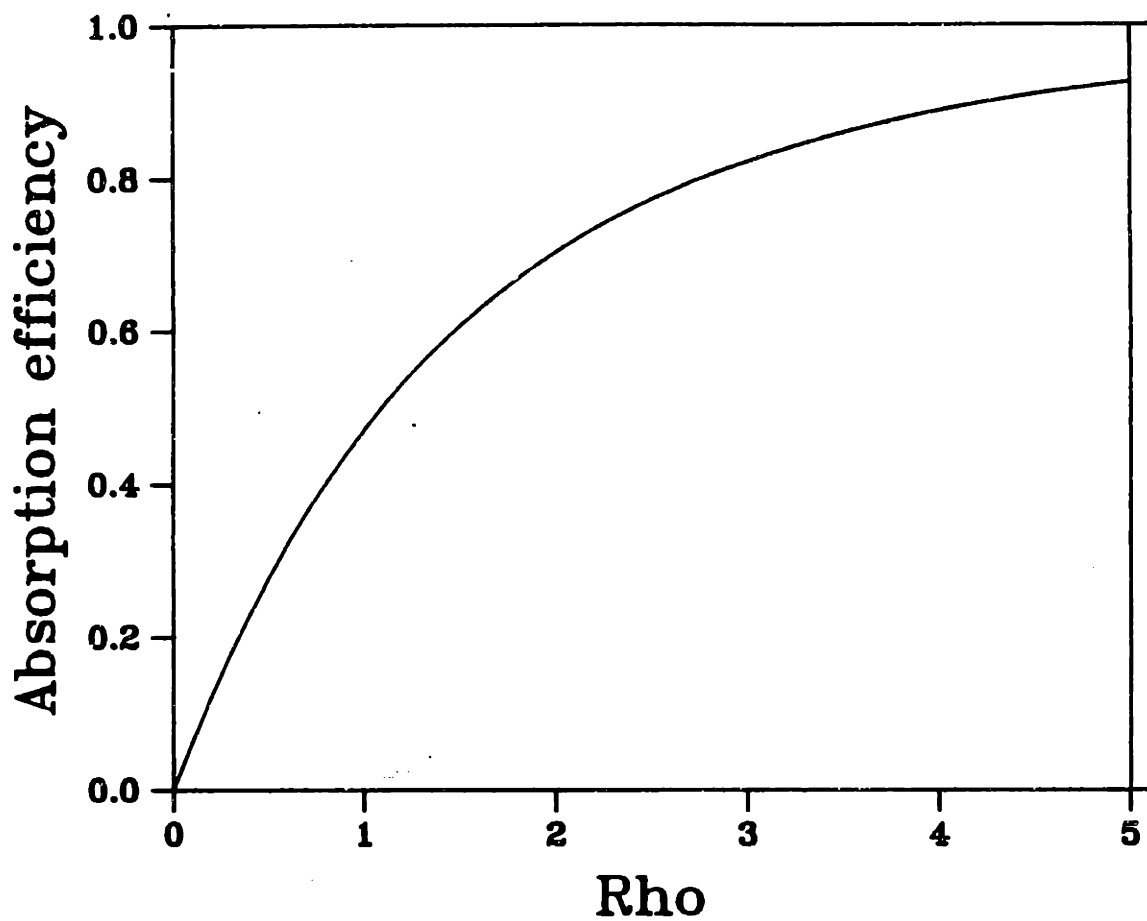
Figure 15. Absorption efficiency (Q_a) as a unique function of the dimensionless parameter, ρ (see text).

of discrete packages containing the same amount of pigment. This effect is discussed in detail in Kirk (1983) and Kirk (1975a).

Using equation (1), Morel and Bricaud (1986) derived absorption efficiency at 675 nm for a range of cell sizes and pigment contents (Fig. 16). They also measured absorption coefficients (a^*), as described in Bricaud et al. (1983), for suspensions of algal cultures representing a range of species and chlorophyll per cell. As discussed above, this a^* can be used to estimate Q_a for an individual cell. Since absorption in the red part of the spectrum at 675 nm is primarily by chlorophyll a with little accessory pigment influence, only chlorophyll a concentration was considered. The theoretical Q_a, which relies on knowledge of an equivalent cell diameter and intracellular chlorophyll a concentration, agrees well with the Q_a based on measured a^* (Fig. 16). For the purpose of this comparison, the chl a specific absorption coefficient used to determine ρ from equations (3) and (4) is the same as for the red peak of chl a dissolved in acetone.

In summary, Morel and Bricaud's (1986) work on a variety of species suggests that single cell absorption can be modeled using Beer's law and can be predicted based on cell size and pigment content. The implications of this for the present work, are that calculations can be made to determine the relationship between absorption efficiency and chlorophyll a concentration for each of the three species. The consequences of changes in absorption efficiency as they affect fluorescence intensity can then be considered.

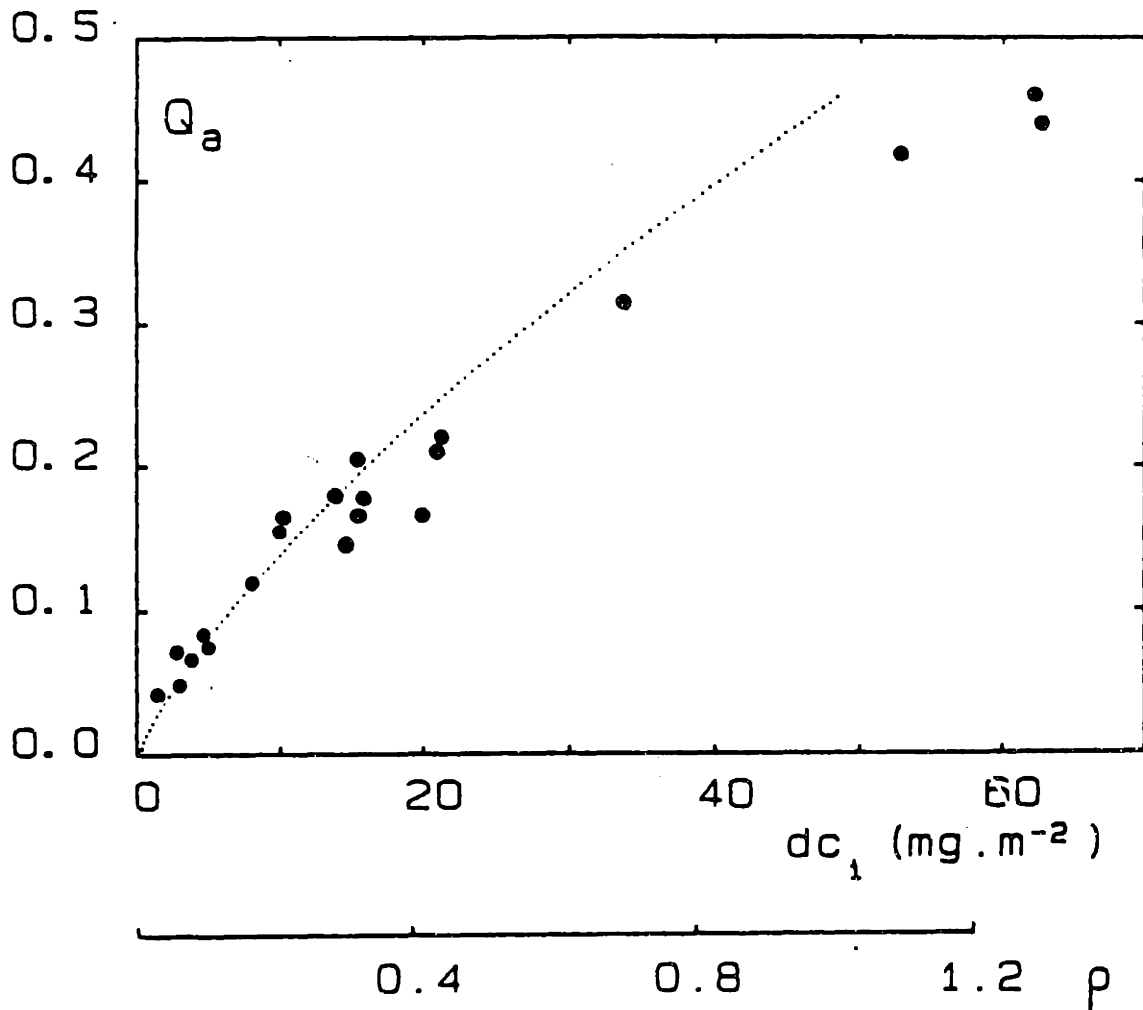Figure 16. Comparison of absorption efficiency (Q_a) as a function of ρ (equation 1) with absorption efficiency computed from experimental values of the chl a specific absorption coefficient at 675 nm. The product dc_i (d - cell diameter, c_i - cellular chl a concentration) is converted to an equivalent ρ scale assuming the absorption coefficient for chl a in acetone. (Morel and Bricaud, 1986).

APPLICATION OF THEORY

For comparison with the fluorescence measurements the absorption efficiency should be calculated at the fluorescence excitation wavelength. Recognizing that the model used by Morel and Bricaud relies on values of c_i and a_i^* which reflect the pigments absorbing at the wavelength of interest, however, these calculations are not easily made. For blue wavelengths, absorption occurs not only by chl a but also by the accessory pigments and it is not possible to determine the appropriate *in vivo* absorption coefficients simply from absorption spectra for pigments extracted in solvents. For this reason, we have made calculations for absorption by chl a at the red peak recognizing that absorption in the blue region is generally stronger and that changes in the relative abundance of pigments will not be reflected in these calculations.

Following the procedure of Morel and Bricaud (1986), the values of ρ and thus Q_a at 675 nm were calculated for each culture (Fig. 17). Values of d_i were determined from the equivalent spherical cell diameter measured on a Coulter Counter and chl a per cell normalized by cell volume was used for c_i. As in the work by Morel and Bricaud (1986), the chl a specific absorption coefficient in acetone was used as the value for a_i^*. It is immediately apparent that the range of values for ρ and Q_a is much smaller for *T. weissflogii* than for either of the other species grown under the same range of conditions. This result is due to the different behavior of cell volume as a function of light intensity between the species. For *H. carterae* and *A. carteri* volume was generally greater in high light cells, while for *T. weissflogii* the opposite was true. As a result, the change in intracellular pigment

Figure 17. Values of absorption efficiency (Q_a) and ρ at the red chl a absorption peak computed for each of the cultures grown at different light intensities. Calculations depend on measured values of equivalent spherical cell diameter and chl a per cell (see text for details).
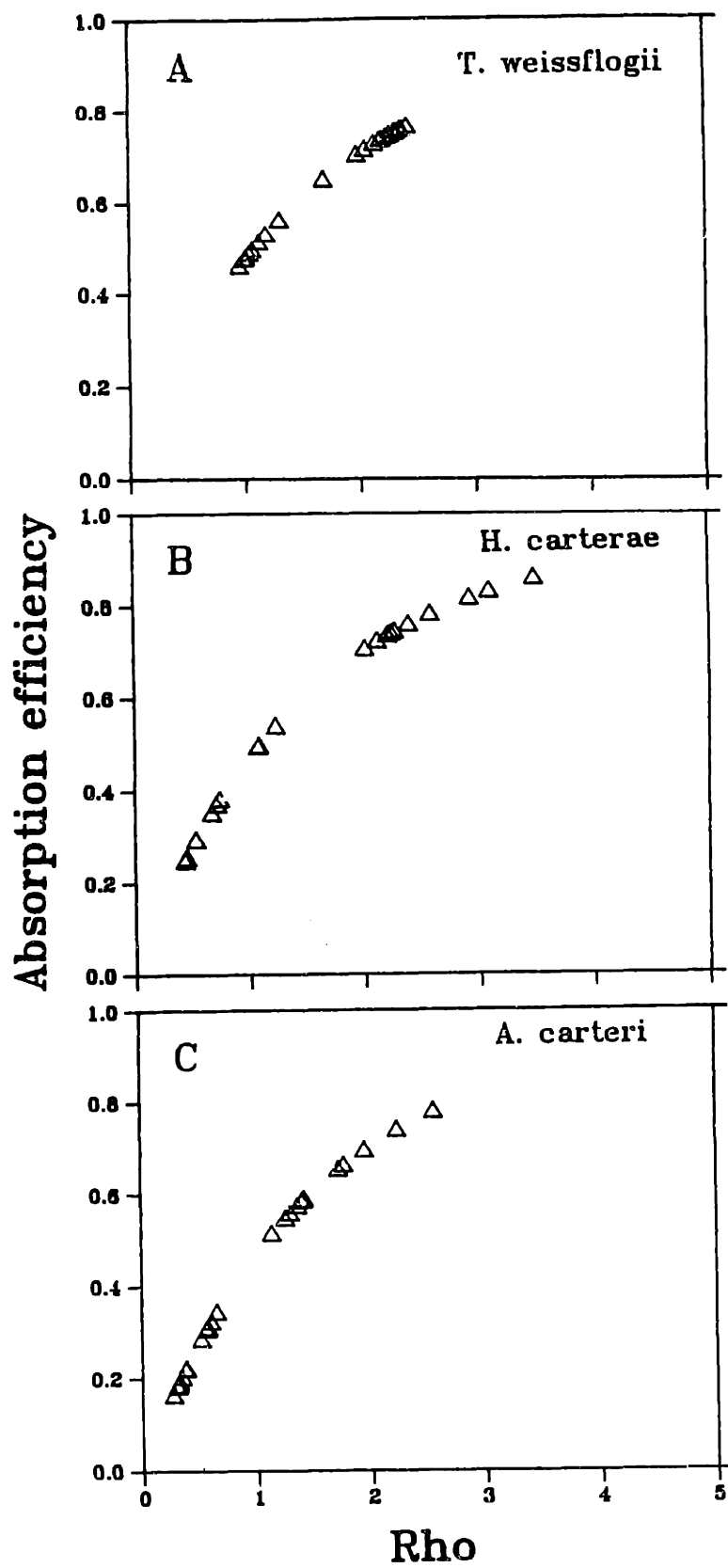
concentration with changing light intensity is not as great in *T. weissflogii* as in the other species.

In order to relate the results for absorption efficiency to parameters relevant to fluorescence some additional computations must be made. Fluorescence intensity depends not directly on absorption efficiency (Q_a) but rather is proportional to the number of photons absorbed. From the definition of absorption efficiency:

$$A_a = s * Q_a \qquad\qquad (4)$$

where s is the geometrical cross-section and the absorption cross-section, A_a, is proportional to the number of photons absorbed. To compare the trends in calculated absorption cross-section to fluorescence , we have assumed that the proportionality constant between absorbed and emitted photons is the same for each of the cultures grown under different light conditions. As discussed in the following section this may not be strictly true if, for instance, the efficiency of electron transfer varies with growth irradiance.

If we now consider absorption cross-section as a function of cellular chl a concentration (Fig. 18), we find that *T. weissflogii* shows a different trend than *H. carterae* and *A. carteri*. For *T. weissflogii*, A_a (and thus photons absorbed) varies linearly with chl a per cell with extrapolated positive intercept while for the other two species, A_a increases linearly at low chl a but climbs less rapidly as chl a continues to rise. Since fluorescence intensity should be proportional to the number of photons absorbed, the trends in absorption cross-section should be reflected in the curves for fluorescence as a function of chl a per cell. Recall that the absorption cross-section is

Figure 18. Absorption cross-section (A_a), the product of absorption efficiency (Q_a) and geometrical cross-section (s) plotted versus chl a per cell for each culture.

Absorption cross-section (μm^2)

A   T. weissflogii

B   H. carterae

C   A. carteri

Chlorophyll a (pg cell^{-1})
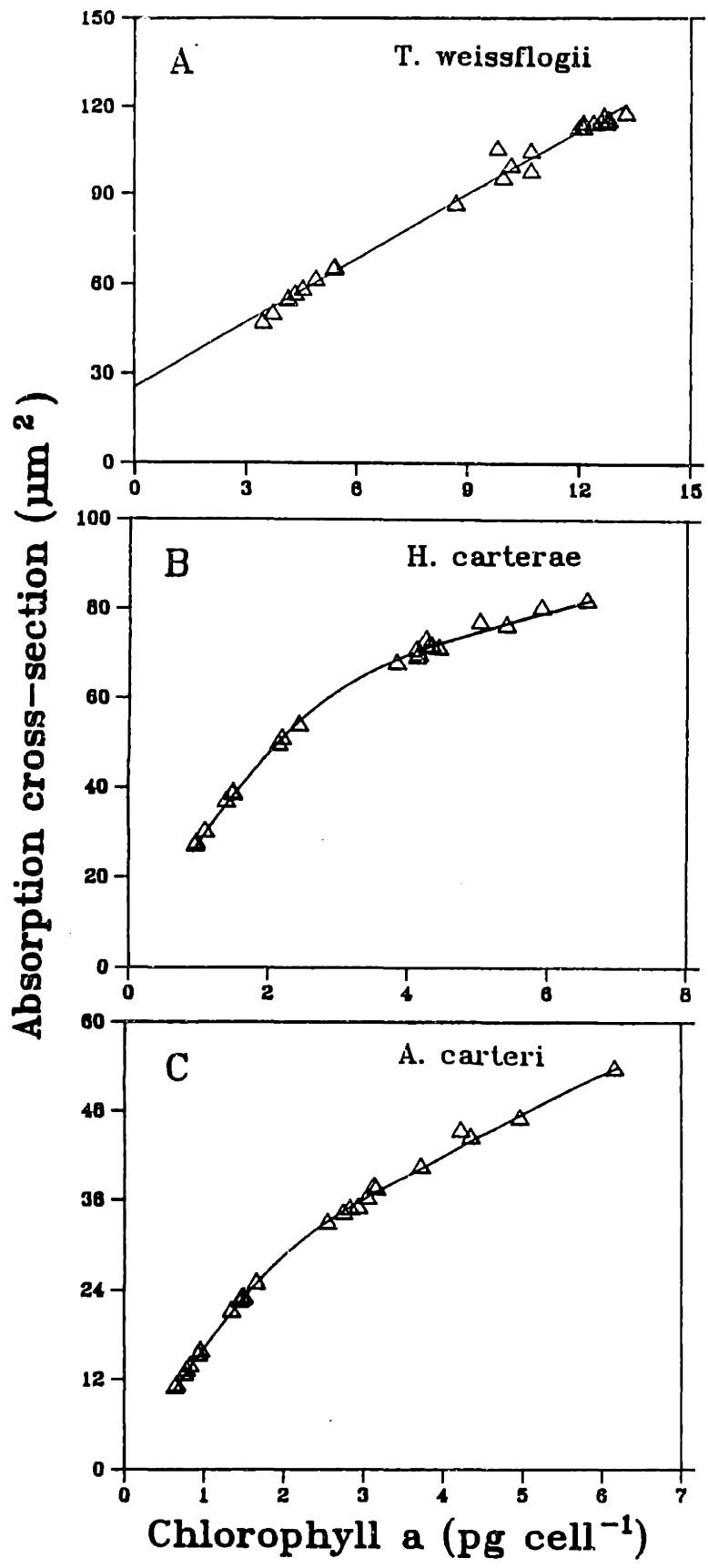
for chl a at 675 nm while the fluorescence measurements are for blue excitation, but the purpose here is only to compare the trends. The similarity of the trends between Figure 18 and Figures 3, 7 and 11 supports the hypothesis that changes in absorption cross-section due to changes in pigment content and cell size are important in predicting fluorescence intensity.

ASSUMPTIONS

The above analysis is admittedly simplified, and is based on a set of assumptions. The effect of particle shape can be significant (Kirk 1975b, 1976) and assuming spherical cells may be particularly a problem for comparing absolute results between species. Moreover, the cells are not homogeneous with respect to pigment distribution. Collins et al. (1985) use the same type of model to apply at the chloroplast level where the assumption of homogeneity is more reasonable. Some details of the effect of pigments being in discrete packages within the cell are also discussed in Morel and Bricaud (1981). The use of the specific absorption coefficients for chl a dissolved in acetone assumes that the chl a molecules behave the same in terms of absorbing capability in this solution as inside the cell. This is not strictly true, however, since pigment molecules within the cell are bound to proteins and will have different properties than when surrounded by solvent molecules.

Despite each of these assumptions the agreement shown in Figure 16 is compelling and argues strongly for the usefulness of the single cell absorption model. Although the validity of the model used by Morel and Bricaud (1986) was demonstrated for a range of species and chl a per cell, none of the cultures used in their verification had values of Q_a

greater than 0.5. In contrast, the calculated values of Q_a for the cultures presented here were often greater than 0.5. These results are expected because the cultures used in Figure 16 were grown at 300 - 400 $\mu Em^{-2}sec^{-1}$ (Morel and Bricaud, 1986), while the data here represent growth over a wider range of light intensities including some as low as 10 - 20 $\mu Em^{-2}sec^{-1}$. It should also be noted that the fit between theory and measurement is not as close for the highest values of ρ as it is for low values (Fig. 16). The deviation suggests that absorption efficiency may saturate at lower values of ρ than predicted from the model. This argues that the above analysis, which is based on the model, may be conservative for predicting effects of intracellular shading in algal cells.

The agreement between measurement and theory for single cell absorption also argues that absorption by chl a is not influenced by accessory pigments at 675 nm. For the blue wavelengths at which absorption is occurring in our analyses of fluorescence, however, accessory pigment absorption cannot be neglected. As mentioned above, for this reason it is difficult to determine values for the specific absorption coefficient (a_i^*) and cellular pigment concentration (c_i) which reflects the effects of all absorbing molecules. However, the trends in absorption cross-section at 675 nm should be related to those for absorption cross-section at blue wavelengths and we have chosen to compare our fluorescence data to these red absorption cross-sections which can be reliably calculated.

Discrepancies between the actual absorption cross-section at blue wavelengths and the absorption cross-section at the red peak result from two sources. First, if changes in the relative contribution of

different pigments to the fluorescence signal occur as a function of growth irradiance, these changes will not be reflected in the red absorption cross-section which is determined solely from chl **a**. However, the effect of this type of change can be evaluated based on pigment concentrations and fluorescence excitation spectra as discussed below. The second factor relates to differences between absorption of blue light versus absorption of red light by chl **a** which are independent of changes in relative pigment abundance. In general, since absorption of blue light is higher, the effects of self-shading on absorption cross-section should only be higher at blue wavelengths (Morel and Bricaud, 1986). This generalization must be recalled when comparing red absorption to blue excited fluorescence.

DISCUSSION

The linearity between fluorescence and chl a observed for *T. weissflogii* suggests a simple relationship between these two parameters. Although a doubling in fluorescence does not necessarily indicate a doubling in pigment content, a given pigment change results in a constant change in fluorescence regardless of the light intensity at which the cells were grown. However, the results for *H. carterae* and *A. carteri* show that the relationship is more complex. In these two species, the same change in fluorescence corresponds to a smaller change in pigment for high light adapted cells than for shade adapted cells. For each species, the yield of fluorescence per chl a molecule increases with growth light intensity.

The effects of pigment changes on fluorescence intensity can be considered to fall in two categories. Although the analysis of the previous section argues strongly for the importance of changes in absolute pigment abundance and cell size in determining these patterns, it is possible that these kinds of fluorescence intensity changes could result from changes in absorption and energy transfer by accessory pigments relative to chl a. We will first consider the effects specifically related to relative pigment composition and then return to absorption cross-section changes which are independent of relative pigment composition.

Changes in the relative contribution to fluorescence by the various absorbing pigments are reflected in the fluorescence excitation spectra and should be related to the pigment composition of the cells. For each species the general trends were the same: as pigment

concentration increased under low light, the amount of accessory

pigments increased relative to chl a (Fig. 19) and the he.:ght of the

fluorescence emission peaks corresponding to absorption by the accessory

pigments increased relative to the chl a peak (see Figs. 4, 8 and 12).

These results suggest that the yield of fluorescence per chl a might

increase in low light cells since there was relatively more contribution

by other pigments under these conditions. We observed the opposite

trend, however, suggesting that the changes reflected in the pigment

ratios and the excitation spectra are not of primary importance in

determining the relationship between fluorescence per cell and cellular

pigment content.

Returning to the absorption cross-section data, we have shown that

a more successful interpretation of the fluorescence-pigment

relationship emerges from considering how the ability of a cell to

absorb photons changes as a function of absolute pigment concentration.

As described in the previous section, it is possible to determine the

dependence of absorption cross-section at the red absorption peak on chl

a concentration for an individual cell. Although flow cytometry

fluorescence was due to blue excitation, we can compare the trends in

fluorescence per cell and in red absorption cross-section to assess

whether changes in the absorption cross-section due to changes in

pigment concentration and cell size are important in predicting

fluorescence. As discussed in the previous section, this is a

conservative comparison since the effects of intracellular shading

should only be higher for blue absorption as compared to red.

While calculated absorption cross-section varied linearly with chl

a per cell for *T. weissflogii*, for the other two species absorption

Figure 19. Molar pigment ratios in *T. weissflogii*, *H. carterae* and *A. carteri* grown over a range of irradiances. A) Chl a : chl c.
B) Chl a : fucoxanthin in *T. weissflogii* and *H. carterae*;
Chl a : peridinin in *A. carteri*.
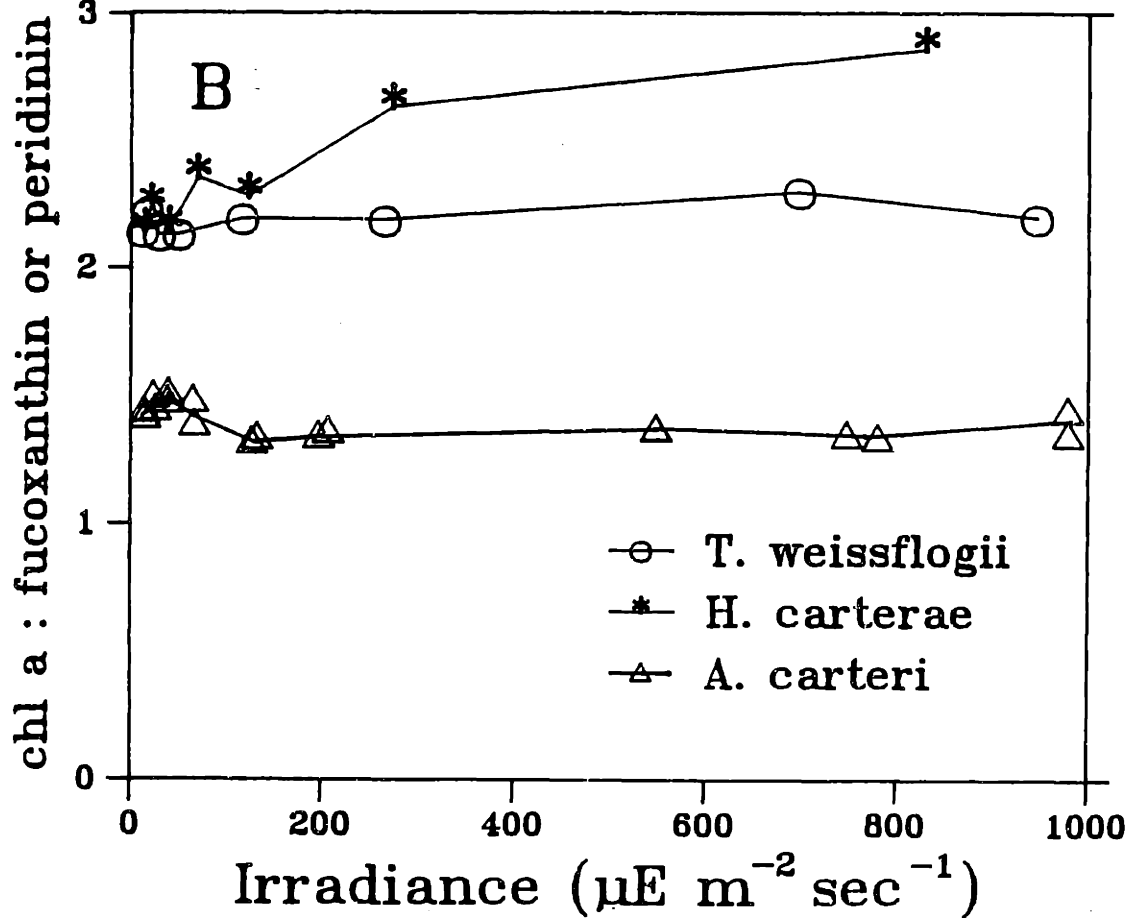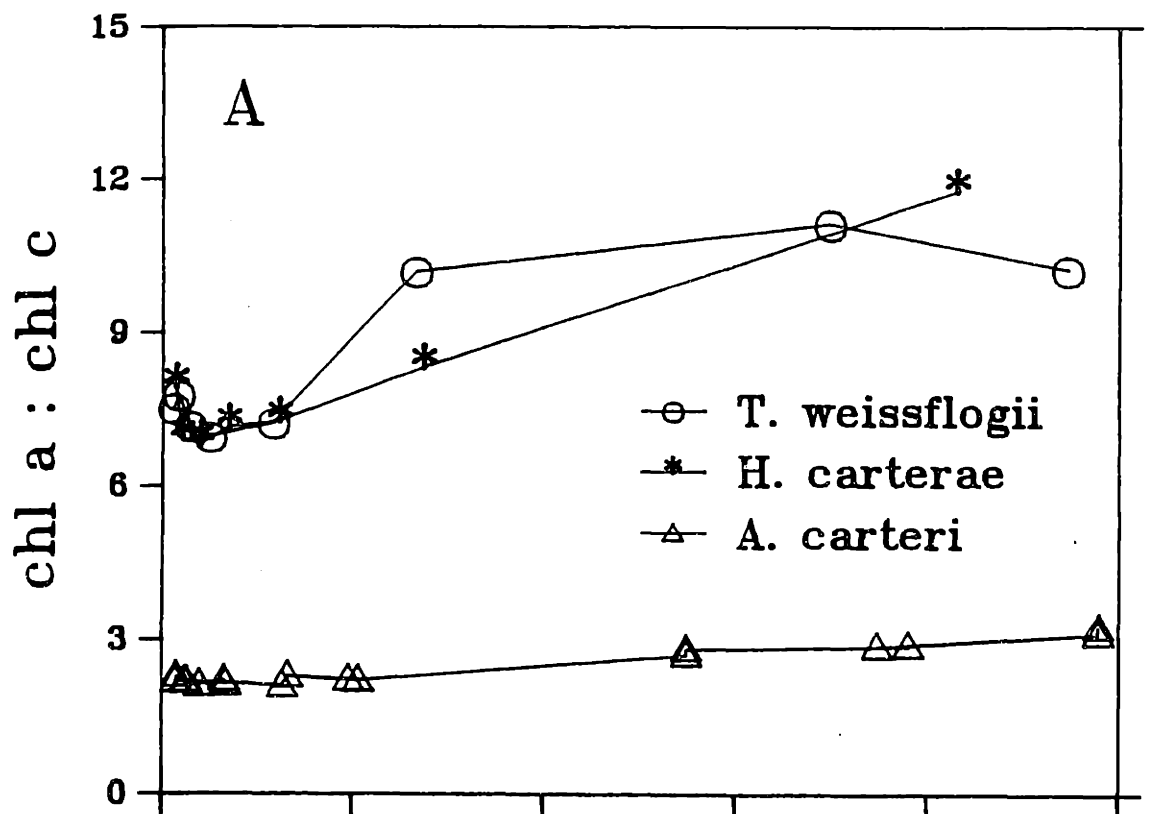
cross-section was directly proportional to chl a per cell at low pigment but increased less rapidly at high pigment content. These trends resemble those for fluorescence (compare Fig. 18 with Figs. 3, 7 and 11), suggesting that intracellular self-shading may be critical in determining fluorescence as a function of pigment content. This explanation accounts for the saturating behavior in fluorescence at high chl a per cell for *H. carterae* and *A. carteri* and the line with extrapolated non-zero intercept for *T. weissflogii* where self-shading occurred but the effect did not vary as widely.

Although chl a increased in low light cells for all three species, cellular volume behaved differently and this difference accounts for the dramatic variation in the shape of the function for absorption cross-section versus chl a per cell. In *H. carterae* and *A. carteri* cell volume generally decreased at low irradiance (see Figs. 6B and 10B). Shading within the cell is more pronounced as pigment concentration increases thus, for these cells, increased mass of pigment and decreased cell volume are compounding effects leading to increased self-shading. In *T. weissflogii*, chl a per cell volume also increased in low light cells due to the increased pigment mass, but the effect is less pronounced because cell volume also increased (see Fig. 2B). The results is that chl a per cell volume in *T. weissflogii* is more constant than in either of the other two species. This accounts for the smaller variation in absorption efficiency for the diatom.

Similar trends as those described here for absorption cross-section as a function of chl a per cell in *H. carterae* and *A. carteri*, have been observed for absorbed irradiance per biomass versus chl a per cell in light- and nutrient-limited cells (Droop, 1985) and for

absorption rate versus chl a per culture volume under light limitation (Schlesinger and Shuter, 1981). These results as well as those described here for bulk *in vivo* fluorescence where absorption or fluorescence per chl a is lower at high chl a can readily be interpreted as due to shading within the cell suspension. This interpretation, however, may be incorrect. As for the results in flow cytometry fluorescence, individual cell measurements which show the same trends suggest that shading within the cell rather than within the suspension may be most important, demonstrating how interpretation based solely on bulk parameters can be limited.

While these results strongly support the hypothesis that knowledge of intracellular shading is critical to understanding the fluorescence-pigment relationship, some questions still remain. It is possible that transfer efficiency of absorbed photons is generally lower in highly pigmented cells. This could contribute to less fluorescence per chl a in these cells. The significance of this effect must be measured by comparing the number of photons absorbed and emitted for individual cells. In addition, to determine more exactly how much effect changes in absorption cross-section have on fluorescence, it is necessary to evaluate absorption cross-section precisely at the wavelength of fluorescence excitation. For instance, the relatively higher chl c to chl a ratio in *A. carteri* (Fig. 19), combined with relatively high blue absorption for chl c (Jeffrey, 1980) may account for the more pronounced flattening of the fluorescence versus chl a at high pigment in this species. If this is correct, it should be reflected in higher blue wavelength absorption cross-sections for *A. carteri*.

Although changes in absorption cross-section predicted based on chl a concentration can be used to explain differences in fluorescence between light and shade adapted cells of the same species, differences in relative abundance of pigments must be considered to account for between species variations. For the same chl a per cell, *A. carteri* consistently had more fluorescence per cell than *T. weissflogii* or *H. carterae* (Fig. 20). *A. carteri* also had more chl c and peridinin relative to chl a than either of the other two species which had similar chl a to chl c and chl a to fucoxanthin ratios (Fig. 19). This can explain the relatively higher fluorescence for the same chl a in *A. carteri* since the other pigments also absorb light of blue wavelengths.

IMPLICATIONS FOR FIELD WORK

From the perspective of applications to field work with flow cytometry these results can be summarized as a set of cautions against over simplified assumptions. While it appears safe to associate higher fluorescence with higher pigment content for a given species, the details of the relationship are not simple. Specifically, fluorescence is not proportional to pigment content and identical fluorescence changes can correspond to different pigment responses even for a single species. For example, the same change in fluorescence corresponds to a bigger change in chl a for low light cells than for high light cells. Between species, it is dangerous even to expect that higher fluorescence indicates more pigment in a cell.

Evaluation of the ability of flow cytometry to differentiate between cells is also relevant to field applications. Under the range
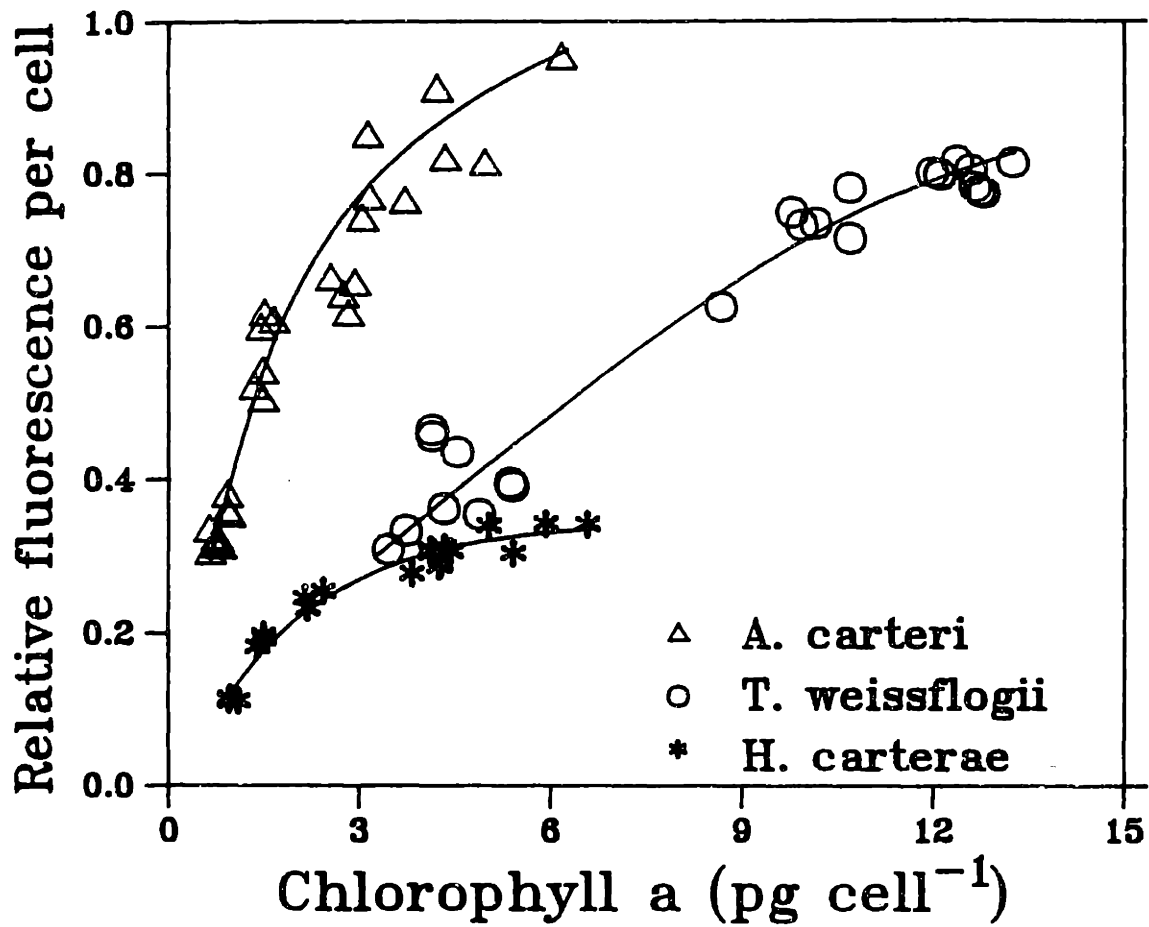
Figure 20. Fluorescence per cell measured on the EPICS flow cytometer
in *T. weissflogii*, *H. carterae* and *A. carteri* as a function of chl a per
cell. Chl a variation was achieved by growing cultures of each species
over similar ranges of light intensity.

of growth conditions observed, it is clear that light adapted cells can be distinguished from shade adapted cells based on fluorescence signals. Conclusive evidence that one parameter is useful awaits investigation of the effects of other growth variables such as temperature and nutrient availability. It may prove that multiple parameters are necessary to distinguish unambiguously high from low light cells. In the present study the ratio of fluorescence excited by 488 nm to fluorescence excited by 515 nm (Ex 488/515) was investigated. The ratio is essentially constant for each species and offers no information about light adaptation (see Appendix A). However, the excitation spectra suggest that other excitation wavelengths, corresponding to the peaks in fluorescence emission, may prove to be more useful. For instance, a higher ratio of fluorescence excited at the chl a peak to fluorescence excited at the chl c peak would be expected for high light relative to low light cells. In addition, such a ratio shows promise toward differentiating between groups of eukaryotic phytoplankton. Even the ratio Ex 488/515 is significantly higher for the diatom than for the dinoflagellate or the coccolithophorid (Appendix A).

Exciting oceanographic applications await the complete description and interpretation of flow cytometry fluorescent signals. One such application is the study of ocean mixing rates elucidated through knowledge of the light history of cells throughout the water column (Chisholm et al., 1988). For a given "tracer" species which could be readily identified at sea, it would be necessary to have detailed knowledge of the light adaptation process as it affects an indicator parameter or group of parameters. The variation in this indicator as a function of light intensity would have to be modelled in a manner

similar to that employed by Falkowski (1983). Some work has been done evaluating the rate constants for changes in chl a with changes in irradiance (Post et al., 1984; Rivkin et al., 1982) and for similar changes in fluorescence (Sakshaug et al., 1987), however, the entire function of fluorescence variation with irradiance must be described. As demonstrated in this work, fluorescence does not necessarily vary linearly with light intensity and even if the rate constant for chl a change is invariant, the corresponding rate constant for fluorescence change would be expected to vary with irradiance.

$$* \quad * \quad * \quad * \quad * \quad * \quad *$$

Flow cytometry has been established as a valuable tool for the characterization of marine particles and their distributions. The extension of applications for this important technology relies on our understanding of the depth to which the optical parameters of individual cells can inform us about properties of the cells and their environment. This study has revealed that the relationship between fluorescence and intracellular pigment content is complex. Although the data is not open to simple interpretation, steps have been taken toward a better understanding of single cell fluorescence signals. This kind of work investigating the details of flow cytometry signals is critical for the promotion of oceanographic applications of this instrumentation.

APPENDIX A

This Appendix consists of data collected in this study which is not specifically relevant to the body of the thesis, but could be useful in future work.

* * * * * * *

The details of changes in fluorescence per cell as a function of changing growth conditions are important for field work directed toward estimating parameters which affect growth from fluorescence properties of the cells. Shown in Figure A.1 is fluorescence per cell as a function of light intensity for three species of eukaryotic algae. It is immediately evident that fluorescence does not change conservatively with irradiance. The plots represent raw data for analysis on the EPICS V flow cytometer which was not directly shown in the body of the thesis.

For each culture analyzed on the EPICS V, forward angle light scatter (FLS) data was collected (Fig. A.2). The interpretation of light scatter signals is important for flow cytometry applications. This data, since it is correlated with fluorescence and cell size measurements, could be useful for beginning to answer questions about what FLS signals can reveal about photosynthetic cells.

As described in (Olson et al., 1988), flow cytometry excitation ratios can be important parameters for distinguishing cells with different pigment characteristics. The data collected in this work for excitation at 488 nm compared to excitation at 515 nm cannot be used to distinguish low light from high light cells in these three species (Fig. A.3). However, it may be useful to differentiate between different species. The ratio is consistently higher for *T. weissflogii* as

compared to *H. carterae* and *A. carteri*. This type of analysis is critical for interpreting field data.

As part of the HPLC analysis several accessory pigments were quantified which were not necessary for the work presented in the thesis (see Table A.1). The variations of these pigments as a function of light intensity, however, are important to other pigment related studies. It may be that changes in the abundance of certain accessory pigments can be used to determine the light history of algal cells in natural samples thus, leading to such developments as the characterization of mixing rates in the upper ocean.

Figure A.1   EPICS fluorescence per cell measured on the EPICS V flow
cytometer (488 nm excitation) as a function of growth light intensity.
Relative fluorescence is expressed in absolute bead units (mean
fluorescence of cell population divided by mean fluorescence of bead
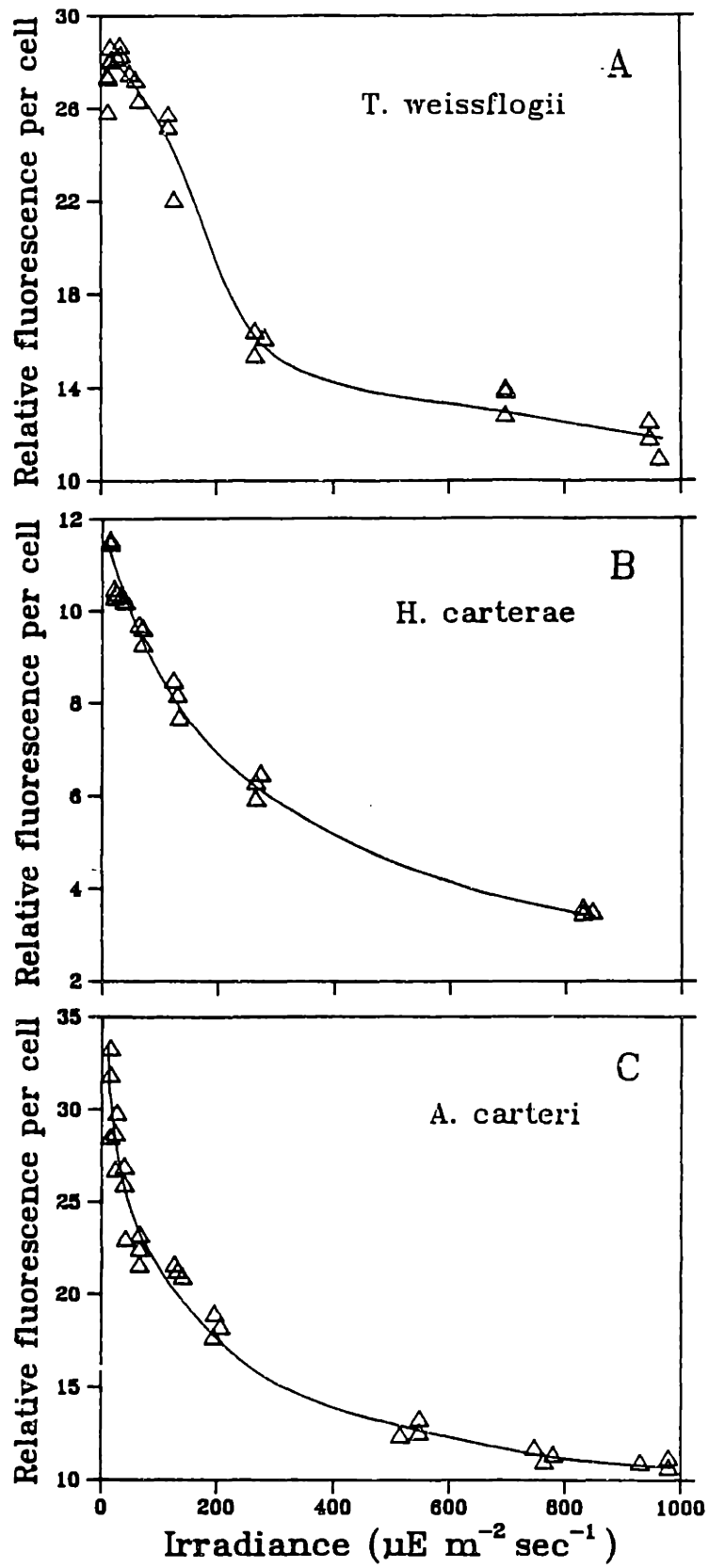distribution) with 2.1 μm Nile Red beads as the standard in all cases.

A T. weissflogii

B H. carterae

C A. carteri

Irradiance ($\mu E\ m^{-2}\ sec^{-1}$)

Relative fluorescence per cell

Figure A.2. Forward angle light scatter (FLS) and cell volume data for cultures grown over a range of irradiances. A) FLS per cell as a function of cell volume. B) Cell volume (measured by Coulter Counter) plotted against irradiance. I)*T. weissflogii*. II)*H. carterae*. III)*A. carteri*. The cell volume data are the same as shown in Figures 2, 6, and 10 in the thesis and are shown here again for reference.

II

A

B
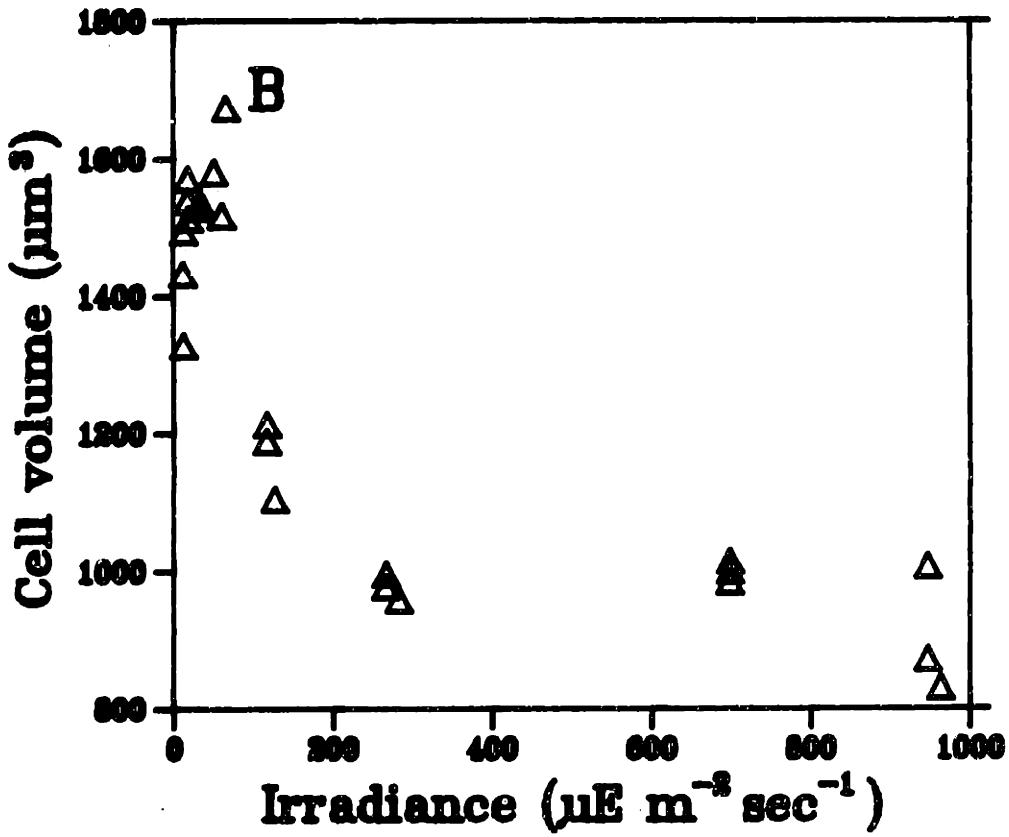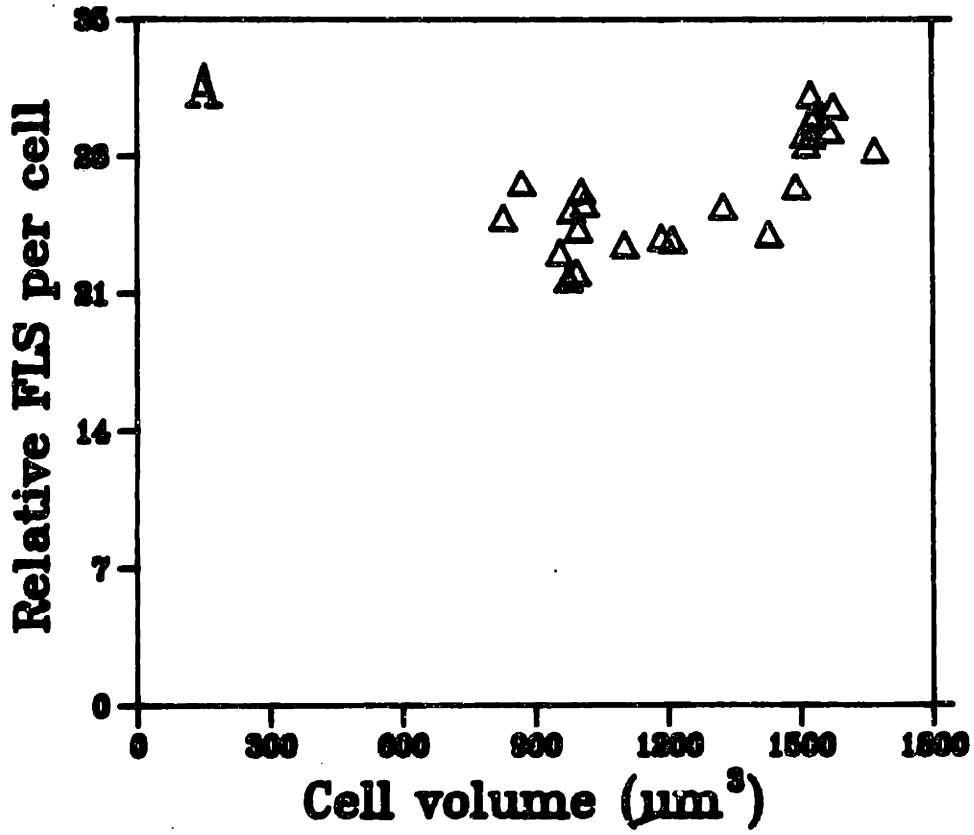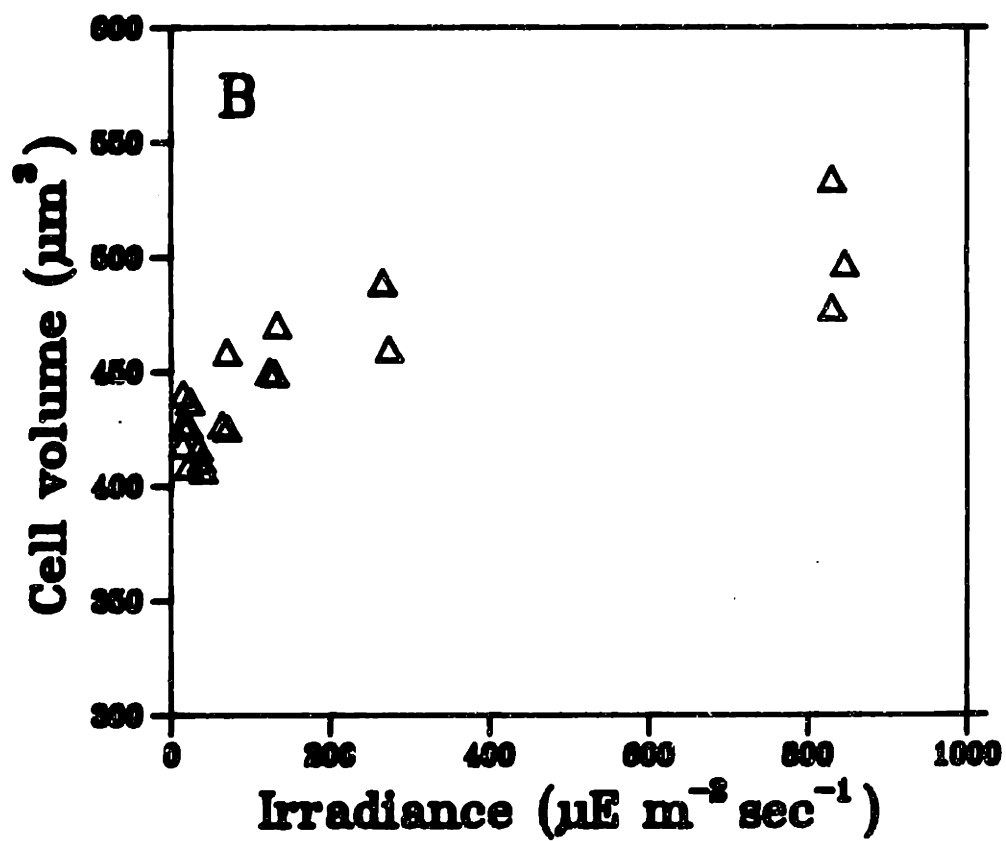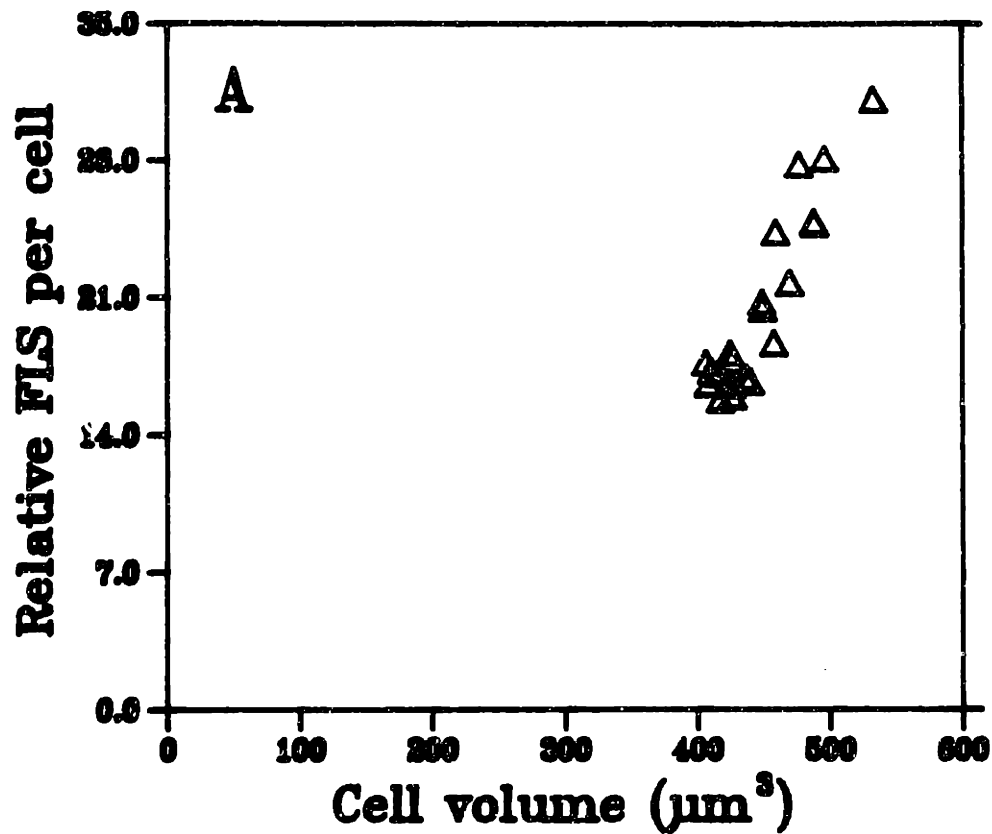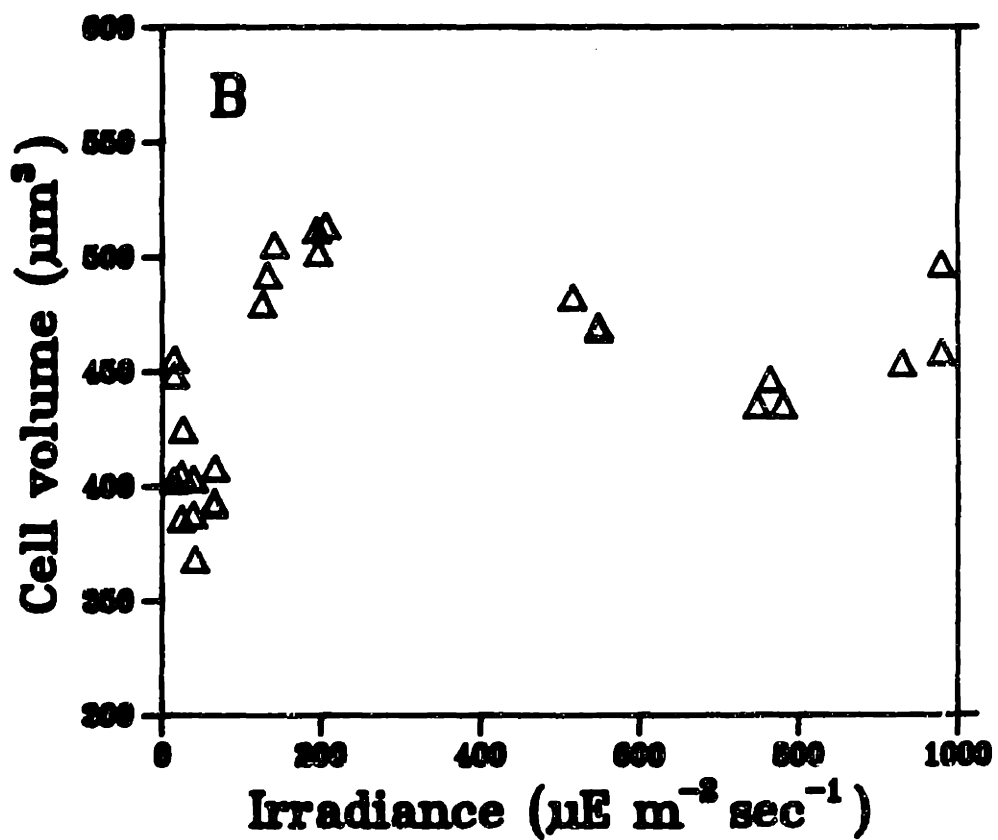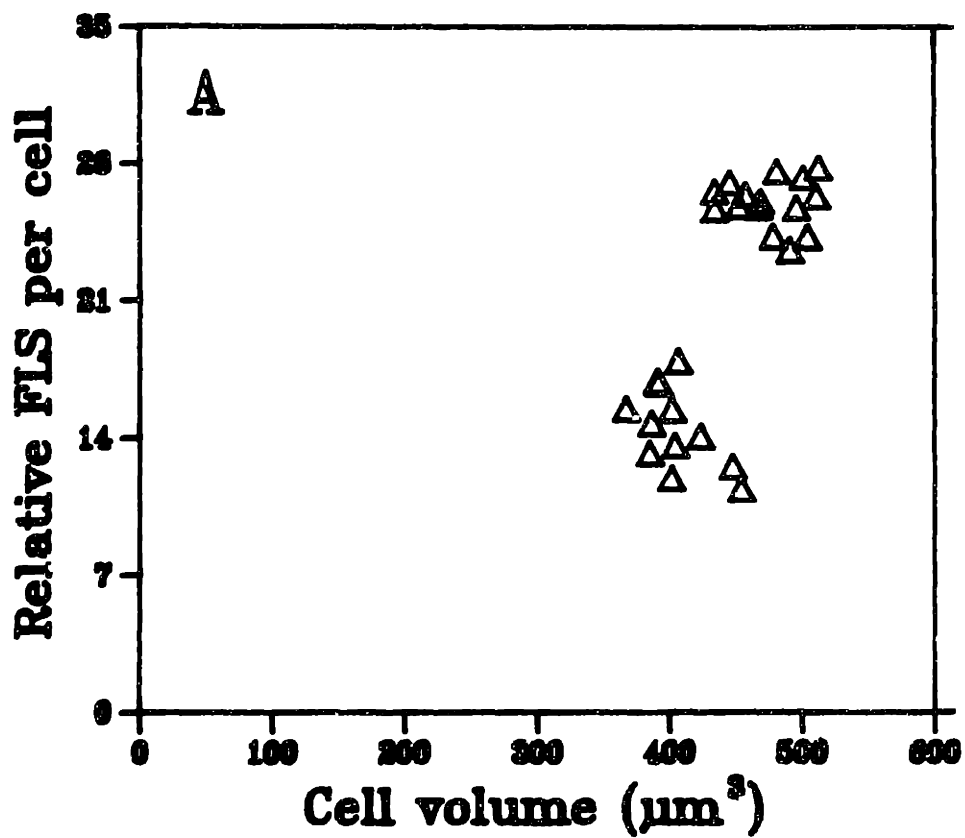
Figure A.3. Ratio of fluorescence emission when excited by 488 nm to fluorescence emission when excited by 515 nm as a function of growth irradiance in each species. All fluorescence data is from analysis on the EPICS V flow cytometer.

Table A.1 Data for additional pigments quantified in HPLC analysis.

T. weissflogii

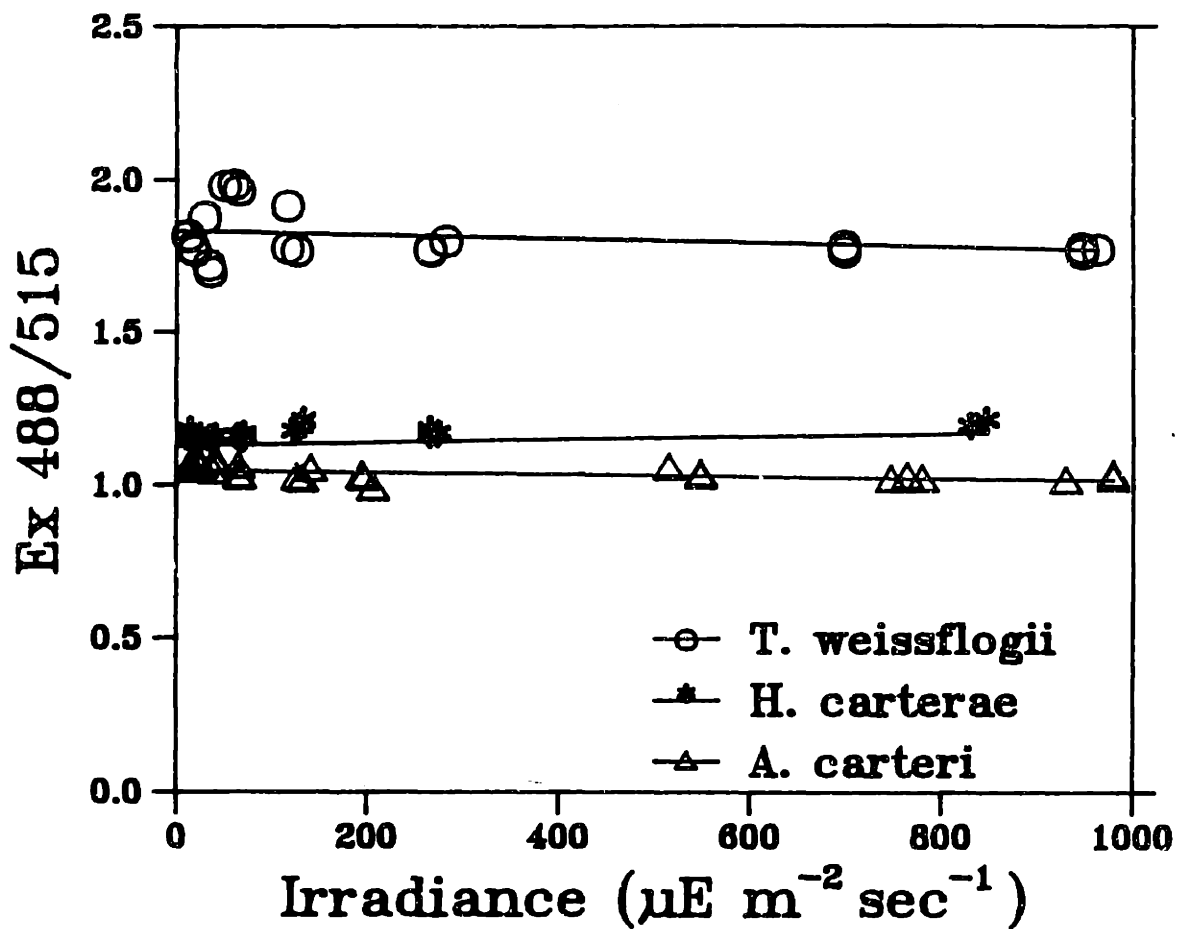| Irradiance ($\mu E\ m^{-2}\ sec^{-1}$)	β-carotene (pg cell^{-1})	diadinoxanthin (pg cell^{-1})	diatoxanthin (pg cell^{-1})
12	0.14	0.55	0.019
17	0.22	0.98	0.028
29	0.15	0.56	0.031
50	0.20	0.60	0.027
120	0.17	0.78	0.055
270	0.12	0.55	0.079
700	0.13	0.75	0.26
950	0.14	0.70	0.33

H. carterae

Irradiance ($\mu E\ m^{-2}\ sec^{-1}$)	β-carotene (pg cell^{-1})	diadinoxanthin (pg cell^{-1})	diatoxanthin (pg cell^{-1})
14	0.15	0.48	0
21	0.13	0.39	0.02
40	0.15	0.41	0.02
70	0.12	0.38	0.02
120	0.08	0.28	0.03
270	0.06	0.25	0.02
830	0.04	0.23	0.02

<u>Table A.1</u> (cont.)

A. carteri

Irradiance ($\mu E\ m^{-2}\ sec^{-1}$)	β-carotene (pg cell^{-1})	diadinoxanthin (pg cell^{-1})	diatoxanthin (pg cell^{-1})	dinoxanthin (pg cell^{-1})
14	0.11	0.68	0.054	0.091
15	0.12	0.81	0.052	0.11
23	0.091	0.59	0.052	0.082
26	0.083	0.66	0.044	0.090
39	0.079	0.52	0.043	0.067
39	0.067	0.52	0.032	0.059
65	0.056	0.44	0.034	0.064
66	0.062	0.47	0.033	0.057
130	0.030	0.50	0.029	0.050
130	0.034	0.51	0.034	0.048
200	0.036	0.54	0.037	0.047
210	0.036	0.52	0.035	0.049
550	0.035	0.54	0.066	0.039
550	0.034	0.53	0.059	0.040
750	0.030	0.48	0.048	0.026
780	0.023	0.52	0.064	0.031
980	0.039	0.54	0.14	0.042
980	0.034	0.59	0.12	0.051

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