

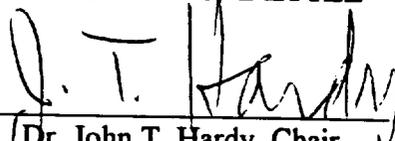
CORAL PIGMENTS: QUANTIFICATION USING HPLC  
AND DETECTION BY REMOTE SENSING

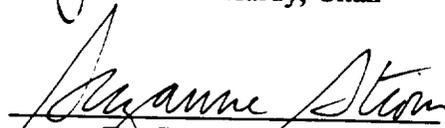
BY  
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Accepted in Partial Completion  
of the Requirements for the Degree  
Master of Science

  
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**MASTER'S THESIS**

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Date July 11, 1995

**CORAL PIGMENTS: QUANTIFICATION USING HPLC  
AND DETECTION BY REMOTE SENSING**

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A Thesis  
Presented to  
The Faculty of  
Western Washington University

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In Partial Fulfillment  
of the Requirements for the Degree  
Master of Science

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Mary C. Cottone  
July 1995

## ABSTRACT

Widespread coral bleaching (loss of pigments of symbiotic dinoflagellates), and the corresponding decline in coral reef health worldwide, mandates the monitoring of coral pigmentation. Samples of the corals Porites compressa and P. lobata were collected from a healthy reef at Puako, Hawai'i, and chlorophyll (chl) a, peridinin, and  $\beta$ -carotene ( $\beta$ -car) were quantified using reverse-phase high performance liquid chromatography (HPLC). Detailed procedures are presented for the extraction of the coral pigments in 90% acetone, and the separation, identification, and quantification of the major zooxanthellar pigments using spectrophotometry and a modification of the HPLC system described by Mantoura and Llewellyn (1983).  $\beta$ -apo-8'-carotenal was found to be inadequate as an internal standard, due to coelution with chl b and/or chl a allomer in the sample extracts. Improvements are suggested, which may result in better resolution of the major pigments and greater accuracy in quantification.

Average concentrations of peridinin, chl a, and  $\beta$ -car in corals on the reef were 5.01, 8.59, and 0.29  $\mu\text{g}/\text{cm}^2$ , respectively. Average concentrations of peridinin and  $\beta$ -car did not differ significantly between the two coral species sampled; however, the mean chl a concentration in P. compressa specimens (7.81  $\mu\text{g}/\text{cm}^2$ ) was significantly lower than that in P. lobata specimens (9.96  $\mu\text{g}/\text{cm}^2$ ). Chl a concentrations determined spectrophotometrically were significantly higher than those generated through HPLC, suggesting that spectrophotometry overestimates chl a concentrations. The average ratio of chl a-to-peridinin concentrations was 1.90, with a large (53%) coefficient of variation and a significant difference between the two species sampled. Additional data are needed before conclusions can be drawn regarding average pigment concentrations in healthy corals and the consistency of the chl a/peridinin ratio.

The HPLC pigment concentration values contribute to the limited database of pigment concentrations in healthy corals, from which quantitative definitions of "healthy"

vs. "bleached" coral may emerge. They also serve as ground-truth, corresponding to fluorescence data collected from the reef at Puako using airborne remote sensing of laser-induced fluorescence. Fluorescence spectra from several overflights using the NASA AOL (airborne oceanographic lidar) system show consistent chlorophyll fluorescence peaks around 685 nm, as well as consistent peaks in the 400-600 nm range which may emanate from granules in the coral tissue. These data, along with results from previous studies of coral fluorescence, suggest that remote sensing of laser-induced fluorescence may become a rapid and efficient means of monitoring coral pigmentation and coral reef bleaching.

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Dr. Cindy Hunter, of the Hawai'i Institute of Marine Biology (Kaneohe, Oahu), graciously introduced us to "her" reef at Puako. Her generosity in sharing her knowledge and good humor made our field sampling much easier and more enjoyable.

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I could not have survived the HPLC process without the help of Terry Meredith, who introduced me to the instrument, kept it running smoothly throughout many long months of calibration and analysis, and, ultimately, trusted me enough to leave me alone with the thing. Clint and Sandy, along with Terry and Bill, were always happy to help me; they made the Instrument Center an unexpectedly pleasant working environment.

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## TABLE OF CONTENTS

ABSTRACT .....	iv
ACKNOWLEDGMENTS .....	vi
LIST OF FIGURES .....	ix
LIST OF TABLES .....	xii
INTRODUCTION .....	1
I. Why quantify coral pigments? Coral bleaching, and the potential use of remote sensing to monitor coral reef pigmentation .....	1
II. Previous methods for quantifying algal pigments .....	4
III. Objectives of this study .....	6
MATERIALS AND METHODS .....	7
I. Field study site and sampling .....	7
II. Laboratory analysis .....	8
A. Surface area determination and preparation of sample extracts .....	8
B. Preparation of pigment standards .....	9
C. HPLC standardization .....	10
D. Coral sample analysis using HPLC .....	11
E. Spectrophotometric analysis of coral sample extracts .....	11
F. HPLC instrumentation and solvent systems .....	12
G. Internal standards .....	12
III. Statistical analysis .....	13
RESULTS .....	14
I. Identification of pigments .....	14
II. Internal standard .....	15
III. Pigment concentrations .....	16
IV. Preliminary remote sensing data .....	18

DISCUSSION .....	18
I. Assessment of pigment extraction and HPLC methods presented .....	18
A. Pigments quantified .....	18
B. Internal standards .....	21
C. Extraction and HPLC methods .....	23
II. Coral pigment concentrations .....	25
III. Coral bleaching and remote sensing .....	28
CONCLUSIONS .....	30
APPENDIX A: Detailed procedures for extraction and quantification of coral pigments using HPLC .....	51
APPENDIX B: Equations for calculating pigment concentrations from spectrophotometric and HPLC data .....	67
APPENDIX C: Absorption spectra .....	70
APPENDIX D: Chlorophyll c / Phaeophytin c .....	77
APPENDIX E: Pigments tested for use as internal standards, and experiments to test for interference with the $\beta$ -apo internal standard .....	81
REFERENCES .....	90

## LIST OF FIGURES

Figure 1. Map of field study and sampling location . . . . .	32
Figure 2. A typical chromatogram from extract of the dinoflagellate <u>Heterocapsa pygmeae</u> (10/1/93) . . . . .	33
Figure 3. Chromatogram from extract of sample 4-7, <u>Porites compressa</u> (6/1/94) . . . . .	34
Figure 4. Chromatogram from extract of sample 4-3, <u>P. lobata</u> (4/6/94) . . . . .	35
Figure 5. Chromatogram from extract of sample 5-2, <u>P. lobata</u> , Hewlett-Packard HPLC system (9/27/94) . . . . .	36
Figure 6. Chromatogram from extract of sample 5-9, calcareous encrusting macroalgae (4/12/94) . . . . .	37
Figure 7. Chromatogram from extract of old coral sample from Station 2, Hewlett-Packard HPLC system (5/2/95) . . . . .	38
Figure 8. Mean peridinin concentrations, by station and for all stations, calculated with and without internal standard (i.s.) data . . . . .	39
Figure 9. Mean chl a concentrations, by station and for all stations, calculated with and without i.s. data . . . . .	40
Figure 10. Mean $\beta$ -car concentrations, by station and for all stations, calculated with and without i.s. data . . . . .	41
Figure 11. Mean pigment concentrations, by station and for all stations (calculated without i.s. data) . . . . .	42
Figure 12. Mean chl a/peridinin concentration ratios, by station and for all stations (calculated without i.s. data) . . . . .	43
Figure 13. Mean coral pigment concentrations, and chl a/peridinin ratios, by species (calculated without i.s. data) . . . . .	44
Figure 14. Mean chl a concentrations, determined by HPLC (no i.s.) and spectrophotometry (equations of Parsons et al. 1984), by station and for all stations . . . . .	45
Figure 15. Three fluorescence spectra from reef at Puako, Hawai'i, generated using the	

NASA AOL system .....	46
Figure 16. Chlorophyll fluorescence (685 nm) with distance along the reef transect at Puako, resulting from UV excitation (355 nm) using NASA AOL system .	47
Figure A1. Example of a calibration curve for $\beta$ -apo-8'-carotenal, 8/3/94 (using data since 3/30/94) .....	63
Figure A2. Chromatogram from calibration mixture .....	64
Figure A3. Example of a calibration curve for peridinin (8/3/94), in which the mass and area values for the pigment of interest are plotted in ratio to those of the internal standard ( $\beta$ -apo) .....	65
Figure C1. Absorption spectrum of pigment I, in ethanol (8/5/93) .....	70
Figure C2. Absorption spectrum of pigment II, in ethanol (1/25/94) .....	71
Figure C3. Absorption spectrum of pigment III, in ethanol (8/12/93) .....	72
Figure C4. Absorption spectrum of pigment IV, identified as phaeophytin c, in HPLC eluent (9/16/94) .....	73
Figure C5. Absorption spectrum of pigment V, in 90% acetone (8/5/93) .....	74
Figure C6. Absorption spectra (in HPLC eluent) for two pigments shown eluting in Figure 15 (chlorophyllide a and chl c) .....	75
Figure C7. Absorption spectra (in HPLC eluent) for three pigments shown eluting in Figure 15 (chl b, chl a allomer, and chl a) .....	76
Figure D1. Chromatogram from <u>Gymnodinium</u> extract, Hewlett-Packard HPLC system (2/8/94) .....	79
Figure D2. Chromatogram from injection of pigment IV into Hewlett-Packard HPLC system (2/8/94) .....	80
Figure E1. Comparison of $\mu$ g internal standard injected, calculated using Method A and Method B, for all HPLC injections (n=63) .....	84
Figure E2. Comparison of $\mu$ g i.s. injected, calculated using Method A and Method B, for coral sample injections only (n=55) .....	85
Figure E3. Comparison of $\mu$ g i.s. injected, calculated using Method A and Method B, for	

non-coral sample injections only (n=8) . . . . .	86
Figure E4. Comparison of $\mu\text{g}$ i.s. injected, calculated using Method A and Method B, for $\beta$ -apo in 90% acetone (n=6) . . . . .	87
Figure E5. Portions of chromatograms from extract of sample 5-2, <u>Porites</u> sp. (9/2/94), with and without i.s. ( $\beta$ -apo) added . . . . .	88
Figure E6. Portions of chromatograms from extract of sample 4-4, <u>P. compressa</u> (8/25/94), with and without i.s. ( $\beta$ -apo) added . . . . .	89

## LIST OF TABLES

Table 1. HPLC specifications . . . . .	48
Table 2. Coral pigment concentration data, calculated without i.s. . . . .	49
Table 3. Summary of areal coral pigment concentration values reported in this and other studies . . . . .	50
Table A1. Approximate absorbance values, at 440 nm, for each of the pigment standards used in HPLC calibration . . . . .	66

## **INTRODUCTION**

### **I. Why quantify coral pigments? Coral bleaching, and the potential use of remote sensing to monitor coral reef pigmentation**

Coral reefs are important biologically, ecologically, economically, and aesthetically throughout the tropics. They provide habitat, in the form of substrate, shelter, and food, for a vast array of marine organisms, forming the basis for an incredibly rich and diverse ecosystem in a nutrient-limited environment. Reefs act as natural breakwaters, protecting shorelines from wave erosion, while creating calm, protected lagoons. These sheltered areas serve as nursery grounds for the juveniles of many marine species, including commercially important fishes and shellfish. Coral reefs are the backbone of tourism, recreation, and fishing industries in many tropical nations, whose economies depend on the health of their reefs. Reefs are also being recognized as a potential source of drugs for fighting human diseases, including AIDS and cancer; this biomedical potential, along with the incredible diversity of life they support, has led many to view coral reefs as the marine equivalent of tropical rainforests (D'Elia et al. 1991; Hoegh-Guldberg 1994).

Since the early 1980s, large-scale bleaching of coral reefs has been observed throughout the world's tropical oceans (Hardy et al. 1992; Hoegh-Guldberg 1994). Some commonly-cited examples are the widespread bleaching in the eastern and central Pacific during the 1982-83 El Niño event, and numerous bleaching events in the Caribbean since the late 1980s (Williams and Bunkley-Williams 1988; D'Elia et al. 1991). Coral bleaching is the loss of symbiotic algae, called zooxanthellae (dinoflagellates, genus Symbiodinium), which normally reside in the coral tissue, giving it its characteristic golden-brown coloration. Bleaching is either the loss of whole zooxanthellae cells from the tissue, or loss of pigment from individual zooxanthellae which remain in the coral (Hoegh-Guldberg and Smith 1989; Kleppel et al. 1989; Szmant and Gassman 1990). The zooxanthellae provide the coral with carbon compounds from photosynthesis, which the coral, in turn, uses for metabolism (Muscatine 1980). Without zooxanthellae, the coral is weakened, it

may fail to grow and reproduce, and it may die (Hoegh-Guldberg and Smith 1989; Goreau and Macfarlane 1990; Jokiel and Coles 1990; Szmant and Gassman 1990; Glynn 1993).

Bleaching can be caused by a variety of stresses, including increased water temperature, increased ultraviolet radiation, decreased salinity, and local nutrient input from point sources of pollution (Goreau 1964; Lesser and Shick 1989; Lesser et al. 1990; D'Elia et al. 1991; Glynn and D'Croz 1991; Hardy et al. 1992; Hoegh-Guldberg 1994). While small-scale, local bleaching occurs frequently and has been recorded since the 1870s, "bleaching events" over large expanses of reef are a recent phenomenon (Williams and Bunkley-Williams 1988; Glynn 1993). Some studies suggest that coral reefs may be good early indicators of ozone depletion and global warming, because of their sensitivity to increased water temperatures and UV radiation (Williams and Bunkley-Williams 1990; D'Elia et al. 1991; Glynn 1993); if so, the monitoring of coral reef pigmentation, as an indication of reef health, gains a new level of global, ecological importance. However, evidence that bleaching is a response to possible global climate change is far from conclusive (D'Elia et al. 1991; Glynn 1993). Even if bleaching is not indicative of world-wide environmental change, the measurement of coral pigmentation on a particular reef, along with (changing) environmental conditions, over time, could further our understanding of the phenomenon of bleaching and aid in our assessment of local reef health.

In the past, coral bleaching has been described mostly qualitatively, and the determination of whether or not a coral head was "bleached" was subjective (Kleppel et al. 1989). In recent years, researchers have studied the possibility of quantifying bleaching, by quantifying the zooxanthellar pigments within the coral tissue. A study by Hardy et al. (1992) proposed the use of remote sensing of laser-induced fluorescence for measuring pigmentation in coral and, thus, monitoring coral bleaching. This study showed that, when irradiated with 532- and 337-nm lasers in the laboratory, corals which were obviously,

visibly bleached (through exposure to high water temperatures) fluoresced less than healthy, unstressed and unbleached corals. Also, corals which had been exposed to temperature stress but were not visibly bleached showed less fluorescence than unstressed corals, suggesting that measurement of fluorescence could be an effective way of detecting bleaching in its early stages, before it is visible, and of quantifying bleaching.

Airborne remote sensing of laser-induced fluorescence is currently used for measuring marine phytoplankton and mapping coastal and terrestrial vegetation (Hoge et al. 1983; Hoge and Swift 1983, 1985), but it has not been used in coral reef studies. In August of 1992, we conducted a pilot field study near Puako, Hawai'i to test the potential for using this technique for monitoring coral pigmentation. Remote sensing from an aircraft would allow for rapid monitoring of large expanses of reef, and would be less labor-intensive than traditional surveying techniques involving SCUBA.

Ground-truth samples were collected, with the intent of using the pigment concentration measurements from the ground-truth samples as a basis of comparison for pigment measurements generated from the remotely-sensed fluorescence data, as a preliminary test of the remote sensing method for monitoring coral pigmentation. The ground-truth coral samples collected during the field study were used in developing the pigment extraction and HPLC methods described in this paper; their pigments were quantified to give an indication of the concentrations of the major coral pigments on the reef at Puako. Because virtually no bleaching was observed on the reef area sampled, the pigment concentrations measured may be assumed to represent those of a healthy, unbleached reef. Few previous studies have quantified zooxanthellar pigments from corals (Gil-Turnes and Corredor 1981; Kleppel et al. 1989); thus, the results presented here will add substantially to the database of "healthy" coral pigment concentrations, providing a baseline from which the term "bleached" can be defined quantitatively.

## **II. Previous methods for quantifying algal pigments**

Numerous past studies have separated and identified (and, in some cases, quantified) algal chlorophyll and carotenoid pigments from different sources. Spectrophotometric and fluorometric methods have been widely used to quantify chlorophylls and general carotenoids in higher plants, algae, and phytoplankton (Jeffrey and Humphrey 1975; Parsons et al. 1984; see Millie et al. 1993 for fluorometric references). Early chromatographic separation techniques included the analysis of zooxanthellar pigments from sea anemones using powdered sugar columns (Strain et al. 1944; Taylor 1967). Paper chromatography has been used to separate the photosynthetic pigments of zooxanthellae from corals and clams (Jeffrey and Haxo 1968). Thin-layer chromatography (TLC) gives more thorough resolution of chlorophyll and carotenoid pigments from marine algae (Jeffrey 1968, 1981) and cultured dinoflagellates (Johansen et al. 1974). Gil-Turnes and Corredor (1981) used medium pressure liquid-solid chromatography to analyze zooxanthellar pigments from corals.

Since the early 1980s, high performance liquid chromatography (HPLC) has become the method of choice for the separation of algal chlorophyll and carotenoid pigments and their derivatives. HPLC can be rapid and highly sensitive, with detection limits of 0.5 ng for carotenoids and 1 ng for chlorophylls, using absorbance detection (Wright and Shearer 1984). Compared with TLC and spectrophotometry, HPLC requires a minute amount of pigment, making it especially convenient when clear, oceanic waters are being analyzed (Wright and Shearer 1984). HPLC allows for the separation of chlorophyll, carotenoid, and xanthophyll pigments which cannot be fully resolved using TLC and spectrophotometry. Inaccuracies of the commonly-used spectrophotometric and fluorometric methods, due to the overlap of the absorbance and fluorescence bands of chlorophylls with those of accessory pigments and degradation products, are eliminated

when HPLC is used (Mantoura and Llewellyn 1983; Bidigare et al. 1985; Millie et al. 1993).

Roy (1987) provides a comprehensive review of HPLC techniques for the analysis of chloropigments. Reverse-phase HPLC, with gradient elution, is recognized as the most efficient technique for complete separation of algal pigments. Mantoura and Llewellyn (1983) first used reverse-phase HPLC, with an ion-pairing reagent, for the rapid (approximately 20 minute) separation and quantification of all major chlorophyll and carotenoid pigments and their degradation products, in algal cultures and natural waters. Their methods have been adapted for subsequent studies of phytoplankton, zooplankton and coral pigmentation (Bidigare et al. 1985; Gieskes and Kraay 1986; Kleppel et al. 1988, 1989; Van Heukelem et al. 1992). Alternative HPLC techniques for the rapid separation of the chlorophylls and carotenoids of marine phytoplankton have also been presented (Wright and Shearer 1984; Wright et al. 1991).

Of the numerous studies cited above, only three discuss the chromatographic separation of zooxanthellar pigments from corals (Jeffrey and Haxo 1968; Gil-Turnes and Corredor 1981; Kleppel et al. 1989). Of these three, only Kleppel et al. (1989) used HPLC to separate and quantify coral pigments, and the procedural details of their HPLC analysis are given not for corals, but for zooplankton pigments (Kleppel et al. 1988). Procedures described for extracting zooxanthellar pigments from coral specimens include: extraction of "whole" pieces of coral (tissue+skeleton, unhomogenized) with methanol (Jeffrey and Haxo 1968), 20% tetrahydrofuran in methanol, or 90% aqueous acetone (Chalker and Dunlap 1981); and extraction, with acetone or 90% aqueous acetone, of coral tissue which has been removed from the skeleton using a jet of compressed air (Gil-Turnes and Corredor 1981; Kleppel et al. 1989).

Of the studies cited above involving HPLC analysis, only Wright et al. (1991) includes consideration of internal standards. Two internal standards were added to the

extracts prior to analysis, and their resolution from the other components of the extracts was reported, but no quantification was performed. Internal standards may be important in improving the precision of HPLC analysis. The use of an internal standard can minimize analytical inaccuracies due to loss of sample during extraction and preparation, as well as variability in measured and injected volumes. Any loss of sample is compensated by an equivalent loss of internal standard; rather than using the absolute peak area of a pigment to quantify that pigment, the ratio of peak area of the pigment to peak area of the internal standard is used in quantification of the pigment (Haefelfinger 1981; Poole and Schuette 1984).

### **III. Objectives of this study**

The objectives of this study were to:

1) develop detailed procedures for the extraction of chlorophyll and carotenoid pigments from coral specimens, the identification of the major coral pigments, and the quantitative analysis of chlorophyll (chl) a, peridinin, and  $\beta$ -carotene ( $\beta$ -car) using reverse-phase HPLC, including the use of an internal standard;

2) estimate the concentrations of three major zooxanthellar pigments (chl a, peridinin, and  $\beta$ -car) in the corals Porites compressa and P. lobata on an apparently healthy reef, in order to expand the database of areal pigment concentrations of healthy corals and provide ground-truthing for remotely-sensed fluorescence data collected over the same reef area;

3) examine ratios of chl a to peridinin concentrations in the two coral species sampled, and consider the potential use of such ratios for quantifying peridinin indirectly using remote sensing, based on chlorophyll fluorescence;

4) compare the concentrations of chl a generated using HPLC analysis with concentrations of chl a determined spectrophotometrically;

5) assess the potential viability of airborne remote sensing of laser-induced fluorescence for monitoring coral pigmentation.

## MATERIALS AND METHODS

### I. Field study site and sampling

A field study was conducted near Puako, off the north west coast of the island of Hawai'i, from August 25-27, 1992 (Figure 1). The reef at Puako was chosen because it is the site of previous coral studies by Dr. Cindy Hunter (Hawai'i Institute of Marine Biology, Kaneohe, Oahu) and because of its proximity to a remote sensing project being conducted by NASA over the southern Pacific Ocean. The NASA P-3 research aircraft flew several passes over a 1.8-km-long transect line (marked with orange buoys), at a speed of 123 m/s and an altitude of approximately 200 ft. Fluorescence was actively induced in the underlying substrate using pulsed lasers (frequency-doubled Nd:YAG lasers) which emitted 355- and 532-nm light every 10 nanoseconds, and the upwelling fluorescence between 370 and 719 nm was recorded with a 32 channel radiometer. Pulses resulted in a fluorescence spectrum sample approximately every 5 m along the transect. The "footprint" was approximately 0.3 m in diameter. For details on the instrumentation of the NASA airborne oceanographic lidar (AOL) system, see Hoge and Swift (1983).

Ground-truth samples were collected at 1-m intervals along 6 10-m-long transect lines ("stations"), laid out at several locations along the longer aircraft transect (Figure 1). Most stations were at a depth of approximately 8 m; station 5 was much shallower, at <2 m. Most samples collected were corals of the genus Porites -- either P. lobata or P. compressa. Occasionally (particularly at the shallower depth) the 1-m mark fell over an area of dead coral or rock and encrusting algae; in such cases, samples of these materials were collected. Samples were collected either by breaking off fragments of coral manually (i.e., for branching samples of P. compressa) or drilling plugs with a pneumatic drill

powered by a SCUBA tank. Samples were immediately placed in labelled plastic, screw-top centrifuge tubes or plastic bags. Once on the surface, the seawater was removed from the centrifuge tubes and bags; they were closed tightly and kept on ice in the dark, then transferred to a 0°C freezer within several hours. Two to three days following collection, they were shipped, on ice, to Western Washington University, where they were stored at -80°C until they could be analyzed.

## **II. Laboratory analysis**

The following is a summary of the pigment extraction and quantification methods used in analyzing the coral samples collected at Puako. Details of the procedures, as well as specifications regarding sample handling, equipment, and chemicals, are given in Appendix A.

### **A. Surface area determination and preparation of sample extracts**

The surface area of each coral sample was determined using the aluminum foil method (Marsh 1970). Attempts to remove the frozen coral tissue from the skeleton using a high-pressure jet of nitrogen (Knap and Sleeter 1984) were unsuccessful. Thus, each sample (tissue and skeleton) was ground using a cast iron mortar and pestle, and its pigments were extracted with 90% aqueous acetone. Two extractions were generally performed, over a period of approximately 15-20 hours. The supernatants from the two extractions for each sample were combined in a volumetric flask and made up to an accurate volume with 90% acetone. The same relative amount of internal standard,  $\beta$ -apo-8'-carotenal (or ethyl  $\beta$ -apo-8'-carotenoate, for the first sample only) was added to each flask and mixed in thoroughly (see below for preparation of internal standards). All samples, as well as pigment standards, were kept cold, protected from light, and handled in an oxygen-free atmosphere (i.e., glove box filled with nitrogen) whenever possible, to prevent pigment degradation.

## B. Preparation of pigment standards

Pure, crystalline chl a and  $\beta$ -car (Sigma Chemical Co.) were purchased for use as calibration standards. Pigments purchased for trial use as internal standards were:  $\beta$ -apo-8'-carotenal ( $\beta$ -apo; Fluka); ethyl  $\beta$ -apo-8'-carotenoate (ethyl  $\beta$ ; Fluka); mesoporphyrin IX dimethyl ester (Sigma); and canthaxanthin (Fluka). Primary standard solutions were prepared from these powdered pigments by dissolving them in 90% aqueous acetone (chl a), ethanol ( $\beta$ -car,  $\beta$ -apo, ethyl  $\beta$ , canthaxanthin), or chloroform (mesoporphyrin).

Peridinin, along with other major pigments, was isolated from cultures of the dinoflagellates Gymnodinium simplex and Heterocapsa pygmaea. The cultures were filtered (Whatman GF/C filters), filters ground with a mortar and pestle, and pigments extracted with 90% acetone. Following centrifugation, the dinoflagellate extracts (supernatants) were concentrated using Sep-Pak C<sub>18</sub> cartridges, and the major pigments were separated using HPLC. 360-450  $\mu$ L of extract (diluted by 50% with nanopure water) were repeatedly injected, and the pigments corresponding to the major peaks on the chromatograms were collected in separate microcentrifuge tubes as they eluted.

The separated pigments were concentrated and transferred into pure solvents using Sep-Pak cartridges. They were identified by: 1) their color; 2) comparing their absorption spectra (generated using a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer, with Perkin-Elmer 3600 Data Station, PETOS operating system, IFL3 software) with published and unpublished spectra and absorption maxima; 3) comparing the dinoflagellate chromatograms with published and unpublished chromatograms of algal extracts; 4) co-chromatography with prepared standards (for chl a and  $\beta$ -car); 5) using an alternate HPLC system with a fluorescence detector to distinguish the dinoflagellate pigments which fluoresce (chlorophylls and chlorophyllides) from those which do not fluoresce (carotenoids). The alternate system is described in section F, below.

### C. HPLC standardization

The concentrations of the primary standard solutions of chl a, peridinin,  $\beta$ -car, and the internal standard were determined spectrophotometrically using Beer's law, equations of Davies (1976), and extinction coefficients given by Mantoura and Llewellyn (1983; see Appendix B). ( $\beta$ -apo was used as the internal standard in the analysis of all but the first sample; see section G, below.) The  $\beta$ -car primary standard required centrifugation before stable spectrophotometric readings could be obtained, due to the presence of undissolved "specks" in the solution. The internal standard solutions required quantitative dilution prior to spectrophotometry (Appendix A). The original, undiluted internal standard solution was added to the coral sample extracts prior to HPLC injection (section A, above), while the quantitatively-diluted standard was used in calibration (see below). All pigment standards were clarified through centrifugation (15000 RPM, for 5 min, at  $<0^{\circ}\text{C}$ ) prior to HPLC injection. In initial trials, filtration, through Gelman FP-450 Vericel membrane filters or S&S nylon membrane filters (0.45- $\mu\text{m}$  pore size) was found to be inadequate for purification of pigment standards and sample extracts prior to HPLC injection, because the filters (particularly the nylon filters) retained substantial amounts of pigment.

Solvent blanks, followed by serial dilutions of each pigment standard, were injected. Calibration curves were constructed by plotting the area under each pigment peak on the chromatogram, against the amount of that pigment injected. Once the calibration curves for the individual pigments were found to be linear and pass through the origin, a series of mixtures of standards were prepared and injected. Each pigment was then considered in ratio to the internal standard (i.s.); calibration curves were created by plotting (area under pigment peak)/(area under i.s. peak) against (amount of pigment injected)/(amount of i.s. injected). Multiple injections of the same amount of i.s. solution were also made, to test the variability in the resulting peak areas.

For "daily" calibration (i.e., calibration on the day that samples were to be analyzed), two or three calibration mixtures were injected (after the concentration of each pigment standard had been determined spectrophotometrically, as described above). If the data points from the daily calibration mixtures "fit" the previously-generated calibration curve, the peak area and pigment concentration data from these injections were added to data from previous injections, and the equation for the regression line through the calibration points was recalculated (Grob 1977). If the daily calibration data points did not fit the curve, a new regression equation was calculated based on the new data points.

#### **D. Coral sample analysis using HPLC**

After a solvent blank and two calibration mixtures had been injected, the coral sample extracts were injected. An aliquot from each sample was clarified through high-speed centrifugation and diluted with nanopure water (2:1, extract:water) immediately before 80  $\mu\text{L}$  were injected. Generally, once 80  $\mu\text{L}$  from each sample had been run, a third calibration mixture was injected at the end of the day, in case chromatographic conditions had changed during the course of analysis.

The peaks corresponding to peridinin, chl a,  $\beta$ -car, and the i.s. on the sample chromatograms were identified. The ratios of the areas under the peridinin, chl a, and  $\beta$ -car peaks to the areas under the i.s. peak were calculated, for each sample. The calibration curves created from the calibration mixtures, and the calibration curve created for the i.s., were then used to determine the amount ( $\mu\text{g}$ ) of each pigment injected, for each sample (see equations in Appendix B). The amount of each pigment in the total volume of a given sample extract was divided by the surface area of the coral sample, to give the concentrations of peridinin, chl a, and  $\beta$ -car in the sample, in  $\mu\text{g}/\text{cm}^2$ .

#### **E. Spectrophotometric analysis of coral sample extracts**

An aliquot (4 mL) of each sample extract was centrifuged, then analyzed spectrophotometrically (Perkin-Elmer Lambda 3B UV/VIS spectrophotometer; VWR

optical glass cuvettes, 1 cm path length). Concentrations of chl a were calculated using the equations of Parsons et al. (1984).

#### **F. HPLC instrumentation and solvent systems**

Pigments were separated using a Waters gradient elution HPLC system (Table 1). Two reverse-phase C<sub>18</sub> columns were used. Initially, a Waters column was used for separating and collecting pigments from dinoflagellate extracts, for calibration of standards and testing of internal standards, for trial runs with some coral extract, and for analysis of pigmentation in the first coral sample. When pigment peaks were found to be too broad and chromatographic separation inadequate, a new Spherisorb silica C<sub>18</sub> column was purchased; the Spherisorb column was used for recalibration with pigment standards and for the analysis of all coral samples except one (see section G, below).

The solvent system was a modification of that described by Mantoura and Llewellyn (1983). The addition of ion-pairing reagent to Solvent A and to extracts prior to injection was found to have no effect on the resolution of chl a, peridinin,  $\beta$ -car, and the i.s., and thus its use was abandoned. Solvent A consisted of 80% methanol and 20% 0.5 M ammonium acetate buffer (Van Heukelem et al. 1992). All solvents were HPLC-grade. Solvents A and B were degassed with helium overnight prior to use.

A Hewlett-Packard Series 1050 HPLC, with fluorescence detector, was used for identifying pigments in dinoflagellate and coral extracts (Table 1).

#### **G. Internal standards**

Several pigments were tested, through multiple HPLC injections, for use as internal standards, but were ultimately rejected. They included mesoporphyrin IX dimethyl ester, canthaxanthin, and ethyl  $\beta$ -apo-8'-carotenoate (ethyl  $\beta$ ). Ethyl  $\beta$  was used as the i.s. for analysis of the first (trial) coral specimen, using the Waters column;  $\beta$ -apo was used in analysis of all remaining samples, due to better resolution from chl a using the Spherisorb column. See Appendix E for further discussion.

### III. Statistical analysis

Outliers in the pigment concentration data were identified using Dixon's Q test and Grubbs test (when  $n > 25$ ).

A paired-sample t-test (2-tailed) was used to test for significant difference between pigment concentration data generated using the i.s., and data generated without the i.s., for each of the pigments quantified by HPLC (peridinin, chl a, and  $\beta$ -car), for each station separately and for all stations combined. The same test was used to test for significant difference between spectrophotometric- and HPLC-generated chl a concentration data, for each station and for all stations. An F variance ratio test was used to determine homogeneity or heterogeneity of variances for concentrations of each pigment, prior to comparing pigment concentrations in Porites compressa specimens with those in P. lobata; subsequently, two-sample t-tests (2-tailed) assuming equal variances were performed to test for significant differences in peridinin, chl a, and  $\beta$ -car concentrations between the two species. An F-test, followed by a t-test assuming unequal variances was used in testing for a significant difference in chl a/peridinin ratios between the species. F- and t-tests were performed using Microsoft Excel 4.0 software.

A Kruskal-Wallis one-way nonparametric analysis of variance was used to test for significant differences in pigment concentrations by station, for each pigment quantified by HPLC, for ratios of chl a/peridinin, and for chl a values determined spectrophotometrically. The nonparametric test was chosen because of differing sample sizes and variances between stations. When the analysis of variance test indicated significant difference, it was followed by a nonparametric multiple range test to determine where the difference lay. The Kruskal-Wallis and multiple range tests were performed using Statistix 4.1 software.

All tests were performed at the 95% probability level ( $\alpha=0.05$ ).

## RESULTS

### I. Identification of pigments

Chromatograms from extracts of the dinoflagellates Heterocapsa pygmeae and Gymnodinium simplex show the same major peaks (Figure 2). The absorption spectra of the collected pigments are presented in Appendix C (Figures C1-C5). The spectrum for pigment IV (Figure C4) was produced by pigment collected from coral extract, rather than extract from the dinoflagellate cultures. No spectrum was produced for pigment VI, because it was collected in very small quantities and was too dilute, even after concentration, to produce an adequate spectrum.

The first major pigment to elute (I) was bright orange in color and was identified as peridinin. Pigments II and III were both yellow, and their absorption spectra are very similar (Figures C2 and C3). Based on the relative sizes of their peaks and their order of elution, Pigments II and III were tentatively identified as dinoxanthin and diadinoxanthin, respectively. The small peak between III and IV may be diatoxanthin; diadinoxanthin and diatoxanthin have been found to interconvert (Goericke and Welschmeyer 1992; Olaizola et al. 1992; Brunet et al. 1993). Pigment V (a bright green pigment) was clearly identified as chl a, and Pigment VI as  $\beta$ -car.

Pigment IV (a green/yellow pigment) was tentatively identified as phaeophytin c, the result of the unintentional acidification of chl c due to the inappropriately low pH of the buffer solution added to Solvent A of the Waters HPLC system. Its absorption spectrum (Figure C4) matches that of phaeophytin c, while the spectrum of pigment "IV" from coral extract injected into the Hewlett-Packard system (Figures 7, C6), which was adequately buffered and did not cause acidification, resembles that of chl c (S. Strom, personal communication). For further discussion, see Appendix D.

## II. Internal standard

Chromatograms resulting from injections of coral extract clearly exhibit the signature peridinin peak, with a retention time (RT) of 8-9 minutes (Figures 3, 4). Chromatograms from extracts of non-coral specimens lack the peridinin peak (Figure 6).

On some sample chromatograms, the internal standard (i.s.) peak seemed to indicate elution of  $\beta$ -apo without interference (Figure 3). However, in other cases,  $\beta$ -apo seemed to co-elute with another compound; the  $\beta$ -apo peak appeared double, or had a "rider" peak (Figures 4, 5). Even when no rider peak was evident, the  $\beta$ -apo peak sometimes appeared larger than expected, based on a qualitative comparison with previous chromatograms, and based on quantitative calculations of the maximum peak area possible, based on the maximum amount of  $\beta$ -apo injected with each sample (see Appendix E). Both the appearance of multiple peaks and the unusually large size of some (single)  $\beta$ -apo peaks suggested co-elution of another compound with the i.s., which would render it useless. Experiments performed to test for interference with the  $\beta$ -apo internal standard are described in Appendix E. The results of these experiments suggest that some compound(s) were co-eluting with  $\beta$ -apo in a significant number of HPLC runs. The interference was more common and of greater magnitude among non-coral (algal) than coral specimens (see Figures E2 and E3).

To identify the interfering compounds, I injected extracts from two old fragments of Porites sp. (an extra piece of sample 1-7, and a piece from Transect 2) into the Hewlett-Packard HPLC system, now equipped with a flow-through cell to a Hewlett-Packard 8452A diode array spectrophotometer. (Instrumentation as described in Table 1, with the addition of the spectrophotometer, and a Shimadzu auto injector SIL-6B with a 2-mL sample loop. A Microsorb C<sub>18</sub> column, 4.6 x 150 mm, 5  $\mu$ m particle size, was used. Solvents and gradient program were derived from Mantoura and Llewellyn 1983; Strom and Bright, unpublished.) An absorption spectrum was produced for each eluting pigment

(Figures C6, C7). Both chl b and chl a allomer were found in both coral samples (Figures 7, C7; also see Figure 5); either, or both, of these pigments could have co-eluted with the  $\beta$ -apo internal standard in the coral samples analyzed.

The variability was determined for four sets of multiple injections of i.s. For each set of injections, the coefficient of variation was calculated for the peak area measurements resulting from five injections of the same amount of i.s. solution. The first three sets of injections involved ethyl  $\beta$  as the i.s., and the Waters HPLC system with the Waters column; the coefficients of variation were 7.7%, 3.2%, and 3.4%. The fourth set of injections involved the dilutions of  $\beta$ -apo in 90% acetone described in Appendix E, and the Waters HPLC system with the Spherisorb column. (These conditions more closely approximated those of the pigments quantified in the coral extracts than did the previous sets of multiple injections.) The coefficient of variation for the peak areas of these five injections was 12.8%.

### III. Pigment concentrations

Because  $\beta$ -apo seemed to be inadequate as an i.s., pigment concentrations were recalculated based on the absolute values of peak areas and amounts of pigment injected, rather than ratios of these values to the i.s. values. However, when the mean (for all stations) "i.s." and "no i.s." coral pigment concentration values were compared, for each of the pigments quantified, no significant difference was found between the "i.s." and "no i.s." data for concentrations of peridinin, chl a, or  $\beta$ -car (Figures 8-10). When each station was considered separately, significant differences between i.s. and no i.s. data were found for chl a at Stations 2, 3, and 5 (Figure 9), and for  $\beta$ -car at Station 2 (Figure 10).

Because of the apparent unreliability of the i.s. used (see Appendix E), and because no significant difference between the "i.s." and "no i.s." data was found when mean pigment concentrations for all coral samples were considered, the pigment concentrations presented here are those generated without the use of the i.s. information.

For all coral samples, the mean peridinin concentration determined by HPLC was 5.01 (SD=1.47)  $\mu\text{g}/\text{cm}^2$ ; the mean chl a concentration was 8.59 (SD=2.56)  $\mu\text{g}/\text{cm}^2$  and the mean  $\beta$ -car concentration was 0.29 (SD=0.11)  $\mu\text{g}/\text{cm}^2$  (Table 2; Figure 11). A significant difference in peridinin concentrations was found between Stations 1 and 4; neither of the other two pigments showed any significant differences between stations (Figure 11).

The mean ratio of chl a to peridinin, for all stations, was 1.90 (SD=1.00; Table 2; Figure 12). While the Kruskal-Wallis nonparametric analysis of variance test indicated a significant difference between stations ( $p<0.05$ ), the subsequent multiple range test found no significant pairwise differences between stations ( $p=0.05$ ).

No significant differences in mean peridinin and  $\beta$ -car concentrations were found between specimens of P. compressa and P. lobata (Figure 13). (Mean peridinin and  $\beta$ -car concentrations in P. compressa were 4.92 and 0.27  $\mu\text{g}/\text{cm}^2$ , respectively; in P. lobata, they were 4.32 and 0.28  $\mu\text{g}/\text{cm}^2$ .) The mean chl a concentration in P. compressa specimens (7.81  $\mu\text{g}/\text{cm}^2$ ) differed significantly from that of P. lobata specimens (9.96  $\mu\text{g}/\text{cm}^2$ ;  $p=0.02$ ; Figure 13). The mean chl a/peridinin ratios for the two species (1.69 for P. compressa; 2.63 for P. lobata) also differed significantly ( $p=0.05$ ; Figure 13). (Note: Only samples which could be clearly identified as either P. compressa or P. lobata were used in calculating the mean values given in this paragraph. Five samples identified only as "Porites sp.," which were used in calculating the overall values given in Table 2 and Figures 11 and 12, were not used in calculating mean pigment concentrations by species.)

The mean chl a concentration, for all coral samples, determined through spectrophotometry was 14.05 (SD=4.09)  $\mu\text{g}/\text{cm}^2$  (Table 2; Figure 14). (No significant differences were found in spectrophotometric chl a values between stations.) Highly significant differences ( $p<0.001$ ) were found between HPLC and spectrophotometric chl a values for Stations 2, 3, 4, and for all stations combined; significant differences ( $p\leq 0.05$ ) were found for Stations 5 and 6. Only for Station 1 was no significant difference found

between HPLC and spectrophotometric chl a values (Figure 14). The spectrophotometric chl a values were, on average (for all samples), 66% higher (SD=23%) than the HPLC chl a values.

#### **IV. Preliminary remote sensing data**

Fluorescence spectra generated from data collected along the reef at Puako using the NASA airborne oceanographic lidar (AOL) system, with UV (355 nm) excitation, show consistent chlorophyll fluorescence peaks around 685 nm (Figure 15). In addition, several consistent peaks were detected in the 400-600 nm range. Differences in the magnitude of chl fluorescence along the transect (Figure 16) seem to correspond to differences in water depth; the greatest chl fluorescence is found near the ends of the transect, where depths ranged from <1 m to approximately 8 m, and the lowest fluorescence is found in the deepest, middle area where the transect crosses the 60 ft (approximately 20 m) contour line (see Figure 1). Thus, the relatively low chl fluorescence detected in the middle of the transect may be the result of the high attenuation of the emitted light as it travels through the water column, rather than actual low chl concentrations along this section of the reef.

### **DISCUSSION**

#### **I. Assessment of pigment extraction and HPLC methods presented**

##### **A. Pigments quantified**

The major light-capturing pigments of dinoflagellates, including zooxanthellae, are chl a, chl c<sub>2</sub>, and peridinin (Taylor 1967; Jeffrey and Haxo 1968; Johansen et al. 1974). Dinoflagellates also contain small amounts of  $\beta$ -car and other accessory pigments, including the yellow xanthophylls diadinoxanthin and dinoxanthin, which do not function in the capture of light for photosynthesis, but may be important in photoprotection and phototactic response (Prézelin 1987). While chl a, chl c,  $\beta$ -car and diadinoxanthin are

present in many marine algal groups, peridinin and dinoxanthin are specific to dinoflagellates (Jeffrey and Haxo 1968).

Chl a is generally used as an indicator of phytoplankton biomass. However, its widespread presence in the water column, in attached macroalgae, and in endolithic algae which may be present in the coral skeleton (see section B, below), can make it inappropriate as an indicator of coral pigmentation. Because the coral skeleton, possibly containing endolithic algae, was ground and extracted along with the tissue, and because the coral samples may have contained small amounts of macroalgae attached to the skeleton or tissue, the extraction method described here may result in an overestimate of areal coral (zooxanthellar) chl a concentrations. However, with some modifications to the methods described (see section C, below), chl a measurements can be made more accurate. Also, with regard to the monitoring of coral pigmentation using remote sensing of laser-induced fluorescence, measurement of chl a is necessary, since other prominent coral pigments (e.g., peridinin) do not fluoresce. As long as a particular fluorescence spectrum can be identified as emanating from coral, as opposed to attached algae on the reef, the quantification of chl a from that fluorescence spectrum could provide a rapid and accurate measure of coral pigmentation (see part III, below).

Peridinin, the only photosynthetic pigment which is specific to dinoflagellates such as coral zooxanthellae, may be an important "signature pigment," for distinguishing coral from non-coral (e.g., macroalgal) specimens. Peridinin, along with other taxon-specific pigments, has been used extensively as a taxonomic marker in studies characterizing phytoplankton communities (Everitt et al. 1990; Strom and Welschmeyer 1991; Barlow et al. 1993; Millie et al. 1993). Also, because peridinin can be used as an indicator of the presence of zooxanthellae in coral, its areal concentration, determined through HPLC, could be used to distinguish healthy, pigmented coral from bleached coral. Peridinin cannot be detected directly through remote sensing of laser-induced fluorescence;

however, discovery of a quantitative relationship between chl a and peridinin concentrations in coral could make possible the estimation of peridinin concentrations through remote sensing.

$\beta$ -car was quantified in this study largely because it was commercially-available in its purified form, for use in HPLC calibration, and its peak was easily identified on preliminary HPLC chromatograms. As with chl a, its concentration may have been overestimated using the methods described here, because of its presence in any endolithic and/or macroalgae extracted along with the coral sample.

Chl c proved to be elusive and unstable. As discussed in Appendix D, chl c in the dinoflagellate and coral extracts seemed to be acidified to phaeophytin c as it passed through the Waters HPLC system, due to inadequate buffering. Because of the acidification problem, and because pure standards for HPLC calibration were unavailable commercially, chl c was not quantified in this study.

Diadinoxanthin and dinoxanthin were also difficult to identify on HPLC chromatograms. Kleppel et al. (1988) showed diadinoxanthin eluting prior to dinoxanthin for the dinoflagellate Amphidinium sp. However, Wright et al. (1991) reported dinoxanthin eluting prior to diadinoxanthin; a similar elution order is suggested here, based largely on the relative sizes of the peaks in question. In all cases cited, the peak identified as dinoxanthin is substantially smaller than that of diadinoxanthin. Using column chromatography, Strain et al. (1944) and Taylor (1967) found an elution order similar to that reported by Wright et al. (1991) and the findings reported here, while Johansen et al. (1974), using TLC, found the elution order to be similar to that found by Kleppel et al. (1988), with diatoxanthin eluting between dinoxanthin and diadinoxanthin. Clearly, dinoxanthin, diadinoxanthin, and diatoxanthin act inconsistently during chromatography. Diadinoxanthin and diatoxanthin have been found to interconvert in response to changes in irradiance and, possibly, other environmental factors (Goericke and Welschmeyer 1992;

Olaizola et al. 1992; Brunet et al. 1993). My decision not to quantify these accessory pigments was based mainly on my inability to distinguish them consistently.

### **B. Internal standards**

An internal standard is used in HPLC to minimize errors due to sample preparation and laboratory technique -- e.g., to correct for the loss of sample during handling. The internal standard should not exist naturally in the substance to be analyzed, but should be chemically similar to the components being analyzed and respond in a similar way. It should not react with any of the other components and should resolve completely from them in HPLC analysis (Poole and Schuette 1984). Since chromatographic conditions vary with different equipment and specifications, the internal standards tested here are likely to respond differently when used with different HPLC systems.

Ultimately,  $\beta$ -apo proved to be inadequate as an internal standard for analysis of coral pigmentation using the method described, because it co-eluted with other substance(s) in the coral extracts during numerous HPLC runs. Interference with the  $\beta$ -apo i.s. is indicated either by visible double peaks on the chromatograms, or by peak areas too large to be explained by natural variability or inaccurate measures of concentration (see Appendix E). Previous studies have identified a peak eluting just prior to chl a in algal extracts as chl a allomer (Wright and Shearer 1984; Wright et al. 1991; Strom and Bright, unpublished). Both chl a allomer and chl b (which also elutes just prior to chl a) were found in coral samples analyzed using the Hewlett-Packard HPLC system; either, or both, of these pigments could have interfered with the  $\beta$ -apo i.s. to cause an increase in its peak area.

The results summarized in Figures E2 and E3 suggest that the compound co-eluting with  $\beta$ -apo is most likely chl b. These results show that the interference with  $\beta$ -apo was more common, and of greater magnitude, on the non-coral chromatograms than on coral chromatograms (see Appendix E). Since the non-coral, or macroalgal, specimens

may contain significant quantities of chl b (which is found in green algae, including the Hawaiian marine species Ulva fasciata, Dictyosphaeria sp., and Codium edule; Fielding and Robinson 1987; Abbott 1974), while the coral specimens should not, we may postulate that the interfering compound, detected most often in the algal samples, was chl b. Of course, chl a allomer may also be present in the algal samples (as well as coral samples); the interfering peak could be a combination of both.

The presence of chl b in the coral extract was unexpected, since zooxanthellae do not contain this pigment. As mentioned previously, the chl b detected may be attributed to endolithic green algae present in the coral skeleton (Hyman 1940; Halldal 1968; Kleppel et al. 1989). Endolithic algae have been found to contain considerable amounts of chl b (Halldal 1968), along with other chlorophyll and carotenoid pigments which are also present in zooxanthellae. Kleppel et al. (1989) attributed chl b found in coral samples to endolithic algae; however, because they extracted coral tissue only (i.e., no skeletal material), it seems unlikely that endolithic algae would have been present in their extracts. Another likely source of chl b in the coral extracts is attached green algae, such as Ulva, Dictyosphaeria, or Codium, which might be colonizing minute areas of the skeleton or tissue where they are able to out-compete or overgrow the coral.

Despite the documented interference with the i.s., no significant difference was found between the coral pigment concentrations calculated with the i.s., and those calculated without the i.s., when all samples from all stations were considered. Such results, without any interference with the i.s., would suggest that no significant loss of sample occurred during handling. In this case, in which interference was evident, perhaps the quantity (and frequency) of the interfering substance was small enough that its effect on the overall pigment concentrations was negligible (although it appeared to be significant for some individual cases). Or, more likely, the difference between the "i.s." and "no i.s." data is masked by a large variability among the results of multiple HPLC

injections (i.e., variability in the method). While the variability among multiple injections of coral extract was not tested, a large (up to 12.8%) coefficient of variation was found among peak areas from multiple injections of the same amount of i.s. solution.

### C. Extraction and HPLC methods

Grinding of the whole coral sample was not ideal, for a number of reasons. First, the grinding of the calcium carbonate skeleton required substantial physical effort and time; ideally, handling should be minimized to prevent pigment degradation. Also, the grinding of the skeleton along with the tissue resulted in a large amount of homogenate, some of which was inevitably lost in the transfer between grinding and extraction containers, potentially resulting in an underestimate of pigment concentrations. The extraction method described also required that the surface area of the sample be determined using the aluminum foil method (Marsh 1970). The aluminum foil method was found to be awkward and time-consuming, especially when working in a glove box and when the coral surface was convoluted. Although its accuracy was not tested through replication, the difficulty of using the method on branching, or even rounded, samples made me doubt its accuracy. I suspect it may be the greatest source of inaccuracy in the determination of pigment concentrations using the method described, and it may be inappropriate for use in conjunction with such a sensitive technique as HPLC.

Another reason that the skeleton should not be ground along with the tissue is the possible presence of endolithic green algae in the skeleton (discussed previously). As noted above, the inclusion of endolithic algal pigments in the sample extract to be analyzed may have resulted in falsely high measures of chl a and  $\beta$ -car per  $\text{cm}^2$  of coral tissue, and may have introduced chl b into the extract, which may have interfered with the internal standard during HPLC.

Ideally, a known surface area of coral tissue should be removed from the skeleton prior to freezing of the sample, and the tissue alone should be analyzed for pigmentation.

The Water-Pik method and the use of a high pressure jet of nitrogen (Knap and Sleeter 1984) have been shown to be effective for removing tissue from unfrozen coral samples. A known surface area of coral tissue can be removed by placing a circular "mask," of known diameter, over the coral tissue and "water-picking" the area inside the circle clean of tissue (Dustan 1979).

While the HPLC system described here did allow for the quantification of peridinin, chl a, and  $\beta$ -car in coral extracts and has, in fact, been used in a subsequent study of coral pigmentation at Western Washington University (Myers 1995), the inadequate resolution of the internal standard ( $\beta$ -apo) from chl b and/or chl a allomer mandates some improvements in the system. Changes in the extraction method, which should minimize the amount of chl b present in the extract, have been discussed above; however, even when such procedural changes were made, Myers (1995) still found a small amount of pigment (chl a allomer?) eluting at approximately the same time that  $\beta$ -apo would be expected to elute. It seems that, either: 1) new internal standards must be identified, which will not co-elute with compounds present in the extract, when the solvent system described here is used; or 2) a new gradient elution solvent system must be tried, to achieve better resolution of the  $\beta$ -apo i.s. A combination of both these changes may be the best solution. When several coral sample extracts were injected into the HPLC system currently being developed by K. Bright and S. Strom at Shannon Point Marine Center, Anacortes, WA (instrumentation described in Table 1, for Hewlett-Packard system; solvent system derived from Mantoura and Llewellyn (1983); ethyl  $\beta$ -apo-8'-carotenoate used as an i.s.), separation of chl b, chl a allomer, and chl a seemed to be improved over the separation provided by the Waters system described here (see Figure 7).

Adequate buffering of the HPLC solvents is essential. My failure to adjust the pH of the buffer in Solvent A may have caused the acidification of chl c to phaeophytin c.

Luckily, chl a does not seem to have been affected. The pH of the buffer should be adjusted to between 7.0 and 7.2 each time the solvent system is used.

## II. Coral pigment concentrations

The coral pigment concentration values for peridinin and  $\beta$ -car reported here fall within the general range of areal concentrations reported in previous and concurrent studies (Table 3). The chl a concentrations reported here are fairly consistent with those reported by Gil-Turnes and Corredor (1981) and Kleppel et al. (1989), but are higher than those reported by Myers (1995). As mentioned earlier, because of the possible inclusion of endolithic and/or attached algae in the coral extracts analyzed, the concentrations of chl a and  $\beta$ -car presented may be an overestimate of actual coral pigment concentrations, while the concentrations of peridinin (which is found only in dinoflagellates) may be more accurate. The higher concentrations of chl a in *P. lobata* than in *P. compressa* specimens (while concentrations of peridinin did not differ significantly) could be due to a greater association of attached algae (and/or endolithic algae) with *P. lobata*, perhaps related to its morphology. Differences in values reported between the different studies are greatest for peridinin, perhaps suggesting that natural, environmental variability in peridinin concentrations is higher than variability in concentrations of the other two pigments.

Since concentrations of photosynthetic pigments in dinoflagellates, including zooxanthellae, are known to vary with depth and light intensity (Prézelin and Matlick 1980; Dustan 1982; Muller-Parker 1987), a range of pigment concentrations is expected among corals sampled from different depths and from sites with differing environmental conditions. Also, different types of zooxanthellae may contain different concentrations of pigments (Glynn 1993). Such variability should be taken into account when defining average pigment concentrations for healthy corals. Further research is needed before conclusions about generalized average pigment concentrations can be drawn. The concentration values for peridinin, chl a, and  $\beta$ -car presented here add to the database of

pigment concentrations in healthy corals. As more data are collected, from healthy as well as bleached reefs, a quantitative definition of "healthy" vs. "bleached" coral, and a delineation of the stages of coral bleaching, may emerge.

Significant differences in pigment concentrations between the stations sampled might be expected if the stations were from different depths, since, as mentioned above, zooxanthellae can photoadapt to irradiance levels at different depths by changing the concentrations of their photosynthetic pigments. Also, the prevalence of attached macroalgae at shallower depths might be expected to affect pigment concentration values by station, if the stations were located at different depths. However, in this study, all stations but one (station 5) were located at approximately the same depth, and only 3 coral specimens were collected from station 5, so the data are insufficient to lead to any conclusions about differences in pigment concentrations with depth. (While all three of the shallow-water coral specimens were P. lobata, the data do not provide evidence that the greater mean chl a concentration found in P. lobata specimens (along with their attached and endolithic algae) is correlated to the predominance of this species at lower depths, since P. lobata specimens were also collected from the deeper stations.) The only significant difference in pigment concentrations between stations occurred for concentrations of peridinin, which were significantly different between stations 1 and 4. The difference must be attributed to natural variability.

The chl a/peridinin ratio values appear variable between stations, and the average value for all stations (1.90) has a high coefficient of variation (53%; see Table 2). However, even though the Kruskal-Wallis analysis of variance test indicated a significant difference between stations, the multiple range test did not identify any pairwise significant differences. This discrepancy in statistical results can be attributed to differences in the powers of the tests. If chl a/peridinin ratios are calculated from the data from previous studies (Table 3), we find that the ratios range from 0.79 (Gil-Turnes and Corredor 1981)

to 2.95 (Kleppel et al. 1989). Myers (1995) reported a mean chl a/peridinin ratio of 1.4. Again, the value reported in this study falls within the range of previously-reported values. Gil-Turnes and Corredor (1981) concluded that, while there were significant variations in total pigment content between the coral species they studied (also found by Myers 1995), there was no significant difference in the proportion of the different pigments (chl a, chl c, peridinin,  $\beta$ -car, and dinoxanthin). However, this study indicates that the chl a/peridinin ratio did vary between the two species studied, because the chl a concentration varied while the peridinin concentration did not; as mentioned previously, the varying chl a concentration may be due to varying amounts of attached and/or endolithic algae between the species, rather than a difference in actual coral chl a concentrations. As with the pigment concentration values themselves, more data are needed before any generalizations can be made about an "average" chl a/peridinin ratio in healthy corals. If peridinin and chl a within the same cell are found to respond differently to environmental conditions, such as changes in irradiance with depth (Prézelin 1987; Trench 1987), then a general, consistent chl a/peridinin ratio does not exist. In such a case, perhaps different chl a/peridinin ratios can be identified for different conditions of irradiance, etc. Again, further research must be conducted before any conclusions can be drawn.

The chl a concentrations determined through spectrophotometry, using the equations of Parsons et al. (1984), were clearly higher than those determined through HPLC (see Figure 14). Numerous previous studies have noted that spectrophotometry overestimates chlorophyll a concentrations because of its inability to distinguish between chlorophylls, accessory chlorophylls, and chlorophyll degradation products such as chlorophyllides and phaeophytins, and because the absorbance bands of chlorophyll may also overlap with those of accessory pigments (Jeffrey 1974; Mantoura and Llewellyn 1983; Bidigare et al. 1985; Sartory 1985; Millie et al. 1993). The degradation products which may interfere with spectrophotometric determination of chl a are probably relatively

less abundant in coral zooxanthellae than in the natural waters which were sampled for most of the previous studies cited, since the zooxanthellae exist in a sheltered, stable environment in the coral host. Still, the data reported here support previous findings that spectrophotometric determination of chl a overestimates chl a concentration, with respect to chl a values determined using HPLC. Chlorophyllide a and chl a allomer were detected in at least some of the coral extracts analyzed (see Figures 5, 7); the presence of these chl a derivatives may be the cause of the overly-high chl a concentrations given by the spectrophotometric equations.

### **III. Coral bleaching and remote sensing**

Since coral bleaching is either the loss of zooxanthellae cells from the coral tissue, or the loss of pigmentation from the algal cells which remain in the tissue (Hoegh-Guldberg and Smith 1989; Kleppel et al. 1989; Szmant and Gassman 1990), bleaching could be quantified in two ways. When bleaching is due to the loss of whole algal cells from the coral tissue (caused by increased temperatures; Hoegh-Guldberg and Smith 1989), it might be quantified by measuring zooxanthellae densities. Past studies have presented population densities of zooxanthellae per coral surface area for normally-pigmented reef-building corals (see Hoegh-Guldberg 1994 for references); further studies in this area might lead to a quantitative definition of "bleached" coral, with respect to zooxanthellae density. When bleaching is due to the loss of pigmentation from zooxanthellae, without a decrease in zooxanthellae density (caused by increased irradiance; Hoegh-Guldberg and Smith 1989), bleaching must be quantified through measurement of pigmentation. If remote sensing of coral fluorescence is to be used for monitoring coral pigmentation and bleaching, baseline pigment concentration data for healthy corals, such as those presented here, are essential; measures of zooxanthellae density would be inadequate.

The study to test the viability of remote sensing of laser-induced fluorescence for monitoring coral pigmentation is still in progress. Fluorescence spectra generated from the remote sensing overflights in Hawai'i show distinct chlorophyll fluorescence peaks around 685 nm, along with numerous peaks in the 400-600 nm range (see Figures 15, 16). Recent studies by Hardy et al. (1992), Mazel (unpublished), and Myers (1995) have demonstrated that the fluorescence spectra of corals irradiated with UV light are clearly distinguishable from those of algae, due to the presence of fluorescence peaks in the 350-550 nm range of the coral spectra. Algal spectra show only chlorophyll fluorescence peaks at approximately 685 nm and higher. The fluorescence at the lower wavelengths may be caused by green pigment granules in the coral tissue, which possibly function in absorbing harmful UV and transforming it into photosynthetically-useable higher-wavelength light (Logan et al. 1990).

Chlorophyll fluorescence can be quantified through the use of mathematical algorithms (Hardy et al. 1992). The ability to distinguish the remotely-sensed fluorescence spectra of corals from those of algae growing on the reef should allow quantification of the chlorophyll of reef corals from their particular fluorescence spectra. If further quantitative study of coral pigmentation elucidates a consistent mathematical relationship between the concentrations of peridinin and chl a in corals, then we may eventually be able to quantify peridinin indirectly through remote sensing. While more field testing and HPLC ground-truthing are necessary, remote sensing of laser-induced fluorescence shows promise as a method for monitoring coral pigmentation and coral bleaching rapidly, based on chlorophyll fluorescence and, possibly, ratios of non-fluorescent pigment concentrations to concentrations of chl a.

## CONCLUSIONS

1) Spectrophotometry and HPLC were used to quantify peridinin, chl a, and  $\beta$ -car in samples of the corals Porites compressa and P. lobata.  $\beta$ -apo-8'-carotenal was found to be unsuitable as an internal standard, because it coeluted with chl b and/or chl a allomer in at least some of the coral extracts. However, no significant difference was found between the pigment concentrations calculated with and without the i.s. data, possibly due to large variability in the results of multiple HPLC injections. The methods described could be improved by: a) removing a known surface area of tissue from the coral skeleton, prior to extracting pigments from the tissue alone; b) using a different internal standard and/or HPLC solvent system, to improve resolution of the pigments; c) consistently adjusting the pH of the buffer solution to 7.0-7.2, to prevent acidification of pigments.

2) The average coral pigment concentrations found on the healthy reef at Puako, Hawai'i were: 5.01 (SD=1.47)  $\mu\text{g}/\text{cm}^2$  peridinin; 8.59 (SD=2.56)  $\mu\text{g}/\text{cm}^2$  chl a; and 0.29 (SD=0.11)  $\mu\text{g}/\text{cm}^2$   $\beta$ -car. The average concentrations of peridinin and  $\beta$ -car did not differ significantly between the two species sampled; however, the mean chl a concentration in P. compressa specimens (7.81  $\mu\text{g}/\text{cm}^2$ ) was significantly lower than that in P. lobata specimens (9.96  $\mu\text{g}/\text{cm}^2$ ). The chl a and  $\beta$ -car values may be overestimates of coral pigmentation, due to their possible inclusion of pigments from endolithic and/or attached algae. The expanding database of pigment concentrations in healthy corals should allow for the development of quantitative definitions of "healthy" vs. "bleached" corals, and a delineation of the stages of coral bleaching.

3) The average chl a/peridinin concentration ratio for all coral samples was 1.90 (SD=1.00). While variability was observed in the data, no significant pairwise differences in the chl a/peridinin ratio were found between the six stations sampled. The mean chl a/peridinin ratio for specimens of P. lobata (2.63) was significantly higher than that for P. compressa samples (1.69), due to a higher mean chl a concentration in P. lobata extracts.

4) Chl a concentrations determined spectrophotometrically were significantly higher than those determined through HPLC.

5) Since fluorescence spectra of corals are distinguishable from those of algae on the reef, and since algorithms exist to quantify chlorophyll fluorescence, remote detection of laser-induced fluorescence, in conjunction with further HPLC ground-truthing, shows promise as a rapid means of monitoring pigmentation in coral reefs. If further research elucidates consistent mathematical relationships between concentrations of chl a and other major pigments such as peridinin, then the non-fluorescent pigments may also be quantifiable through remote sensing.



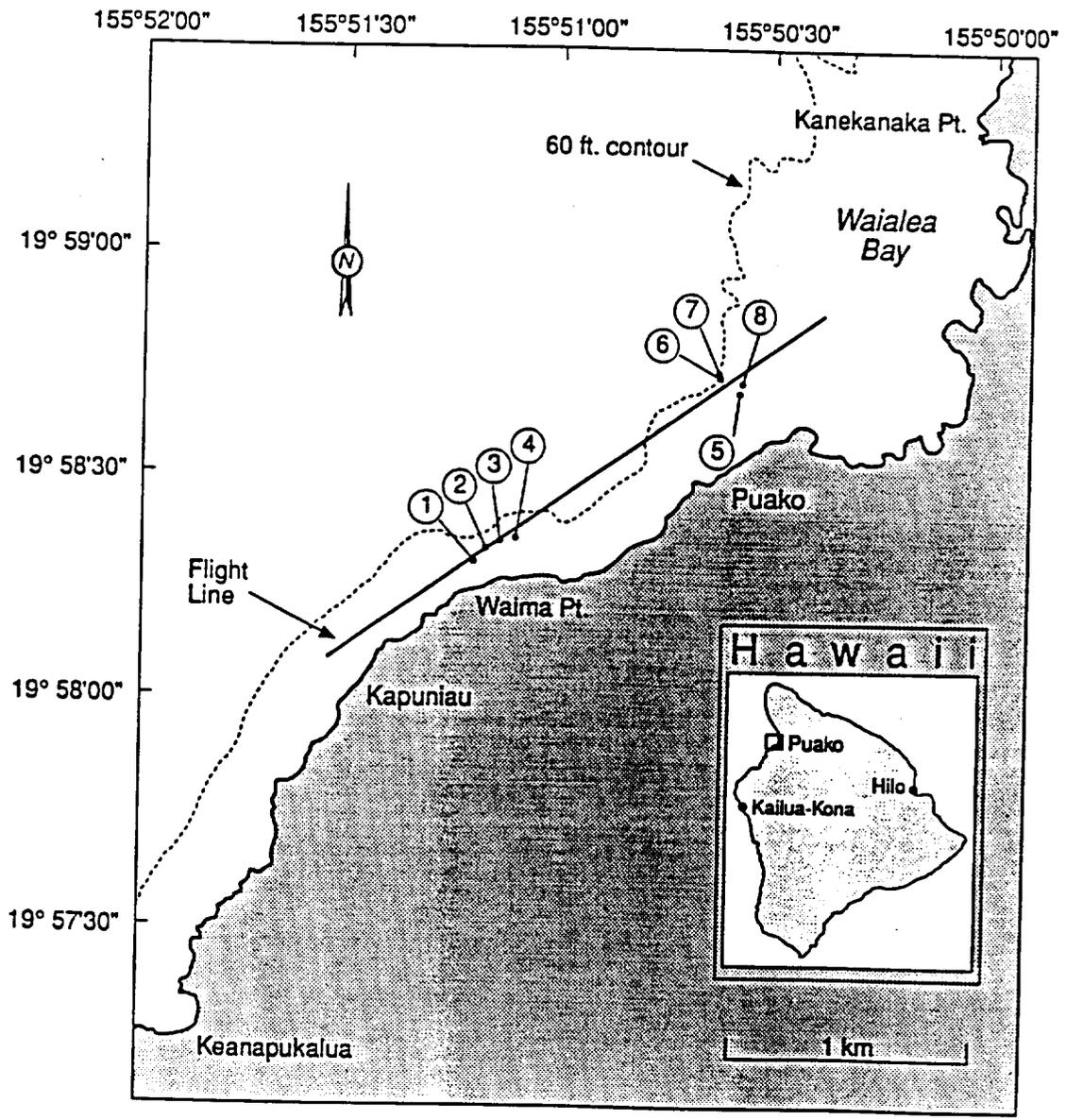


Figure 1. Map of field study and sampling location. Samples were collected from Stations 1 - 6; Stations 7 and 8 were photographic transects only.

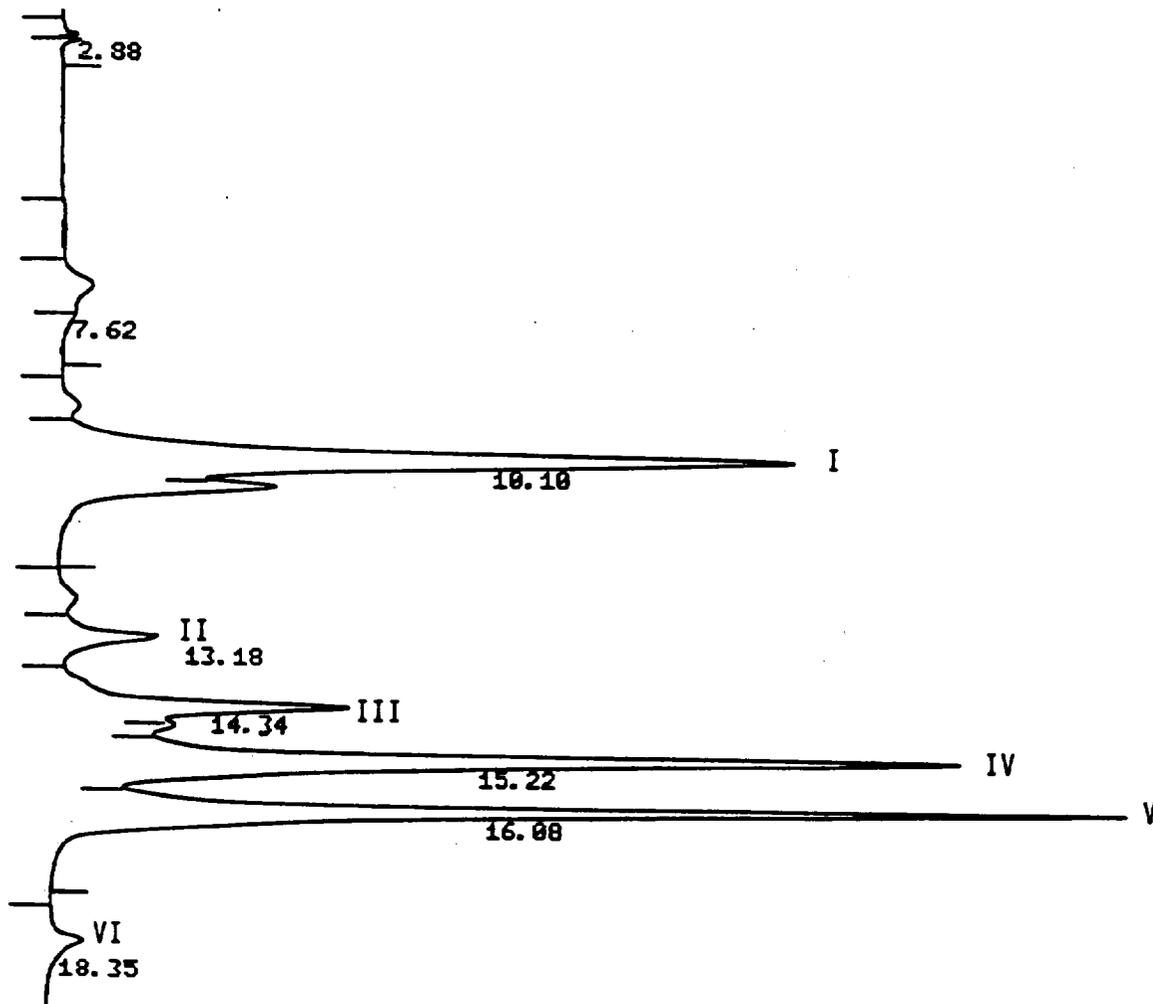


Figure 2. A typical chromatogram from extract of the dinoflagellate *Heterocapsa pygmaea* (10/1/93). Numbers below peaks are retention times (RT), in minutes. I=peridinin; II=dinoxanthin(?); III=diadinoxanthin(?); IV=phaeophytin c?; V=chl a; VI= $\beta$ -car. Waters HPLC system, Waters column.

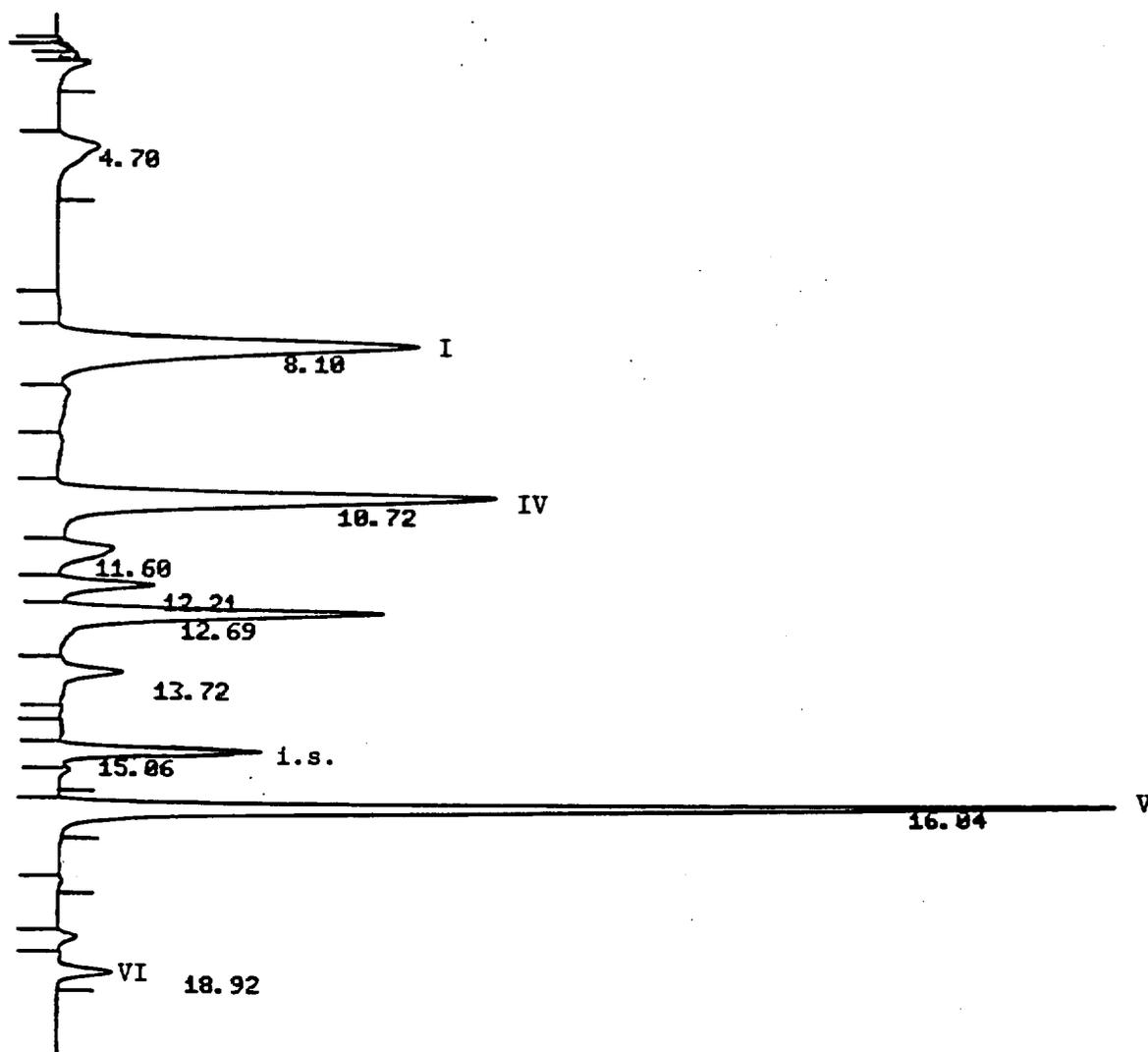


Figure 3. Chromatogram from extract of sample 4-7, *Porites compressa* (6/1/94). Numbers as described for Figure 2; i.s. =  $\beta$ -apo-8'-carotenal. Waters HPLC system, Spherisorb column.

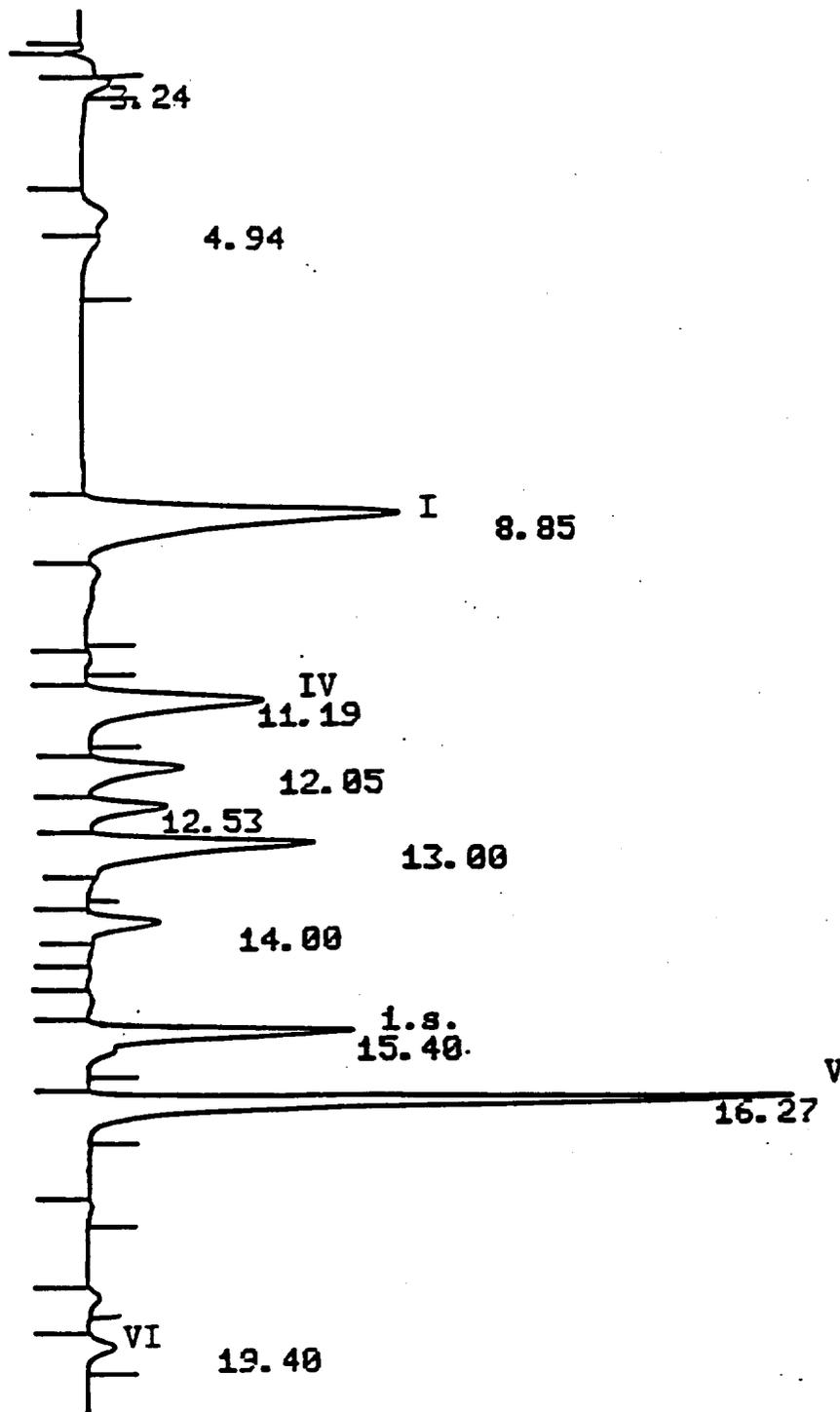


Figure 4. Chromatogram from extract of sample 4-3, *P. lobata* (4/6/94). Numbers as described for Figure 2; i.s. =  $\beta$ -apo. Waters HPLC system, Spherisorb column.

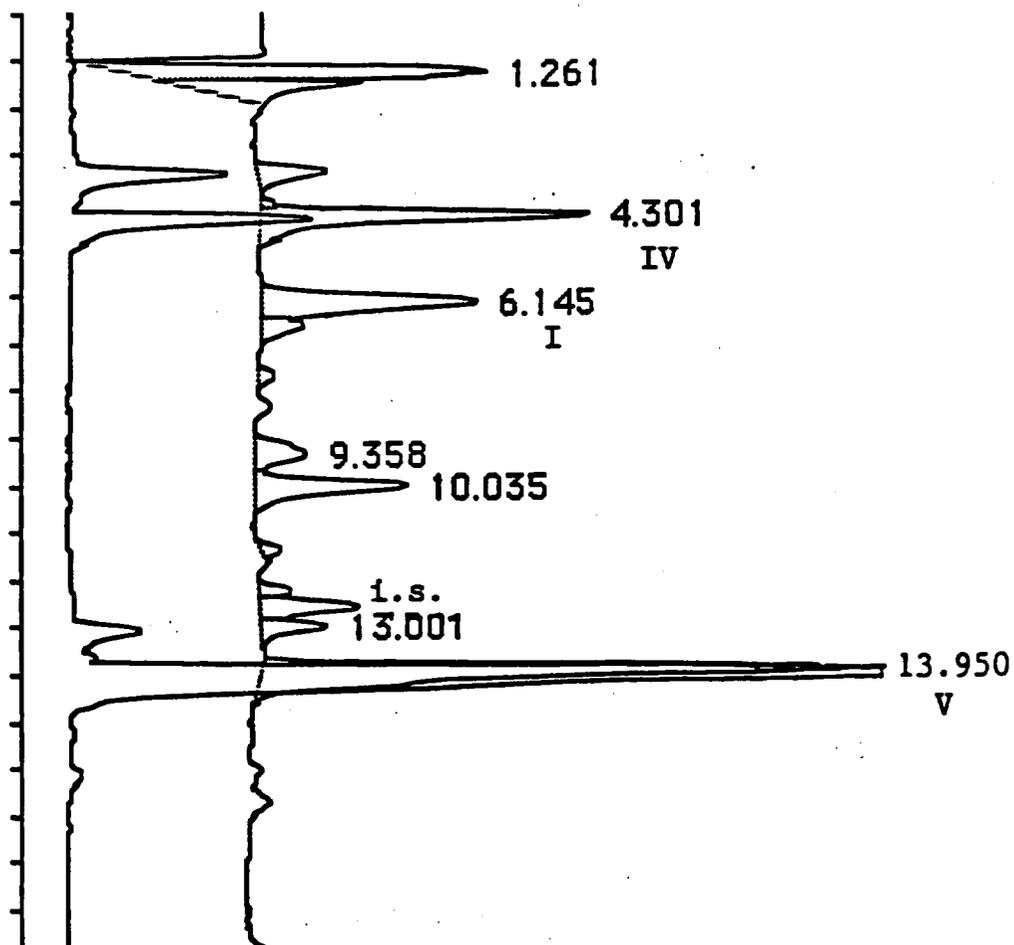


Figure 5. Chromatogram from extract of sample 5-2, *P. lobata*, Hewlett-Packard HPLC system (9/27/94). Numbers as described for Figure 2, except IV=chl c?; i.s. =  $\beta$ -apo. Peak with RT of 13.001 is chl b or chl a allomer (see Results, section II). Peak preceding pigment IV is chlorophyllide a.

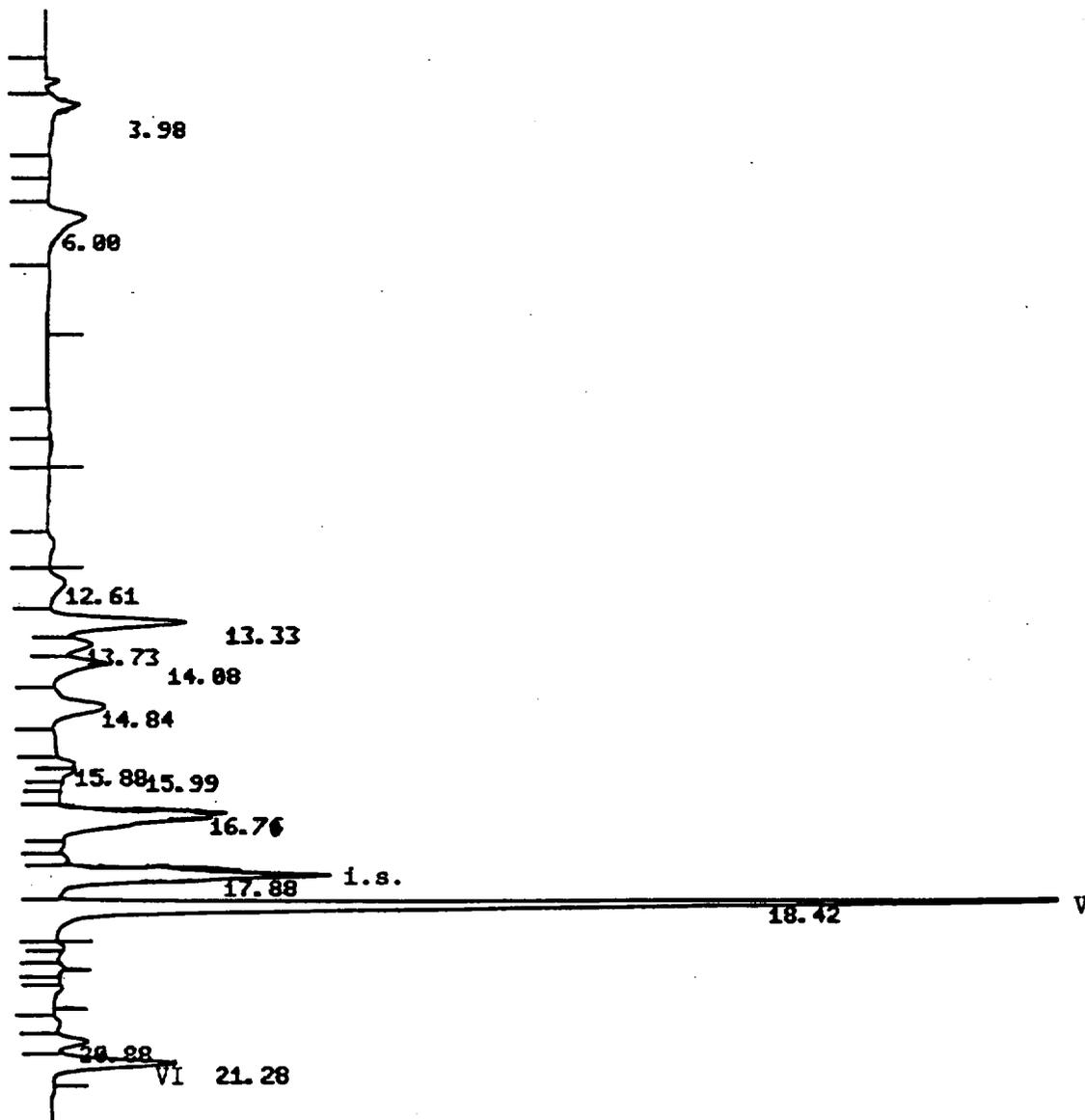


Figure 6. Chromatogram from extract of sample 5-9, calcareous encrusting macroalgae (4/12/94). Numbers as described for Figure 2; i.s. =  $\beta$ -apo. Waters HPLC system, Spherisorb column. Note lack of pigment I, peridinin.

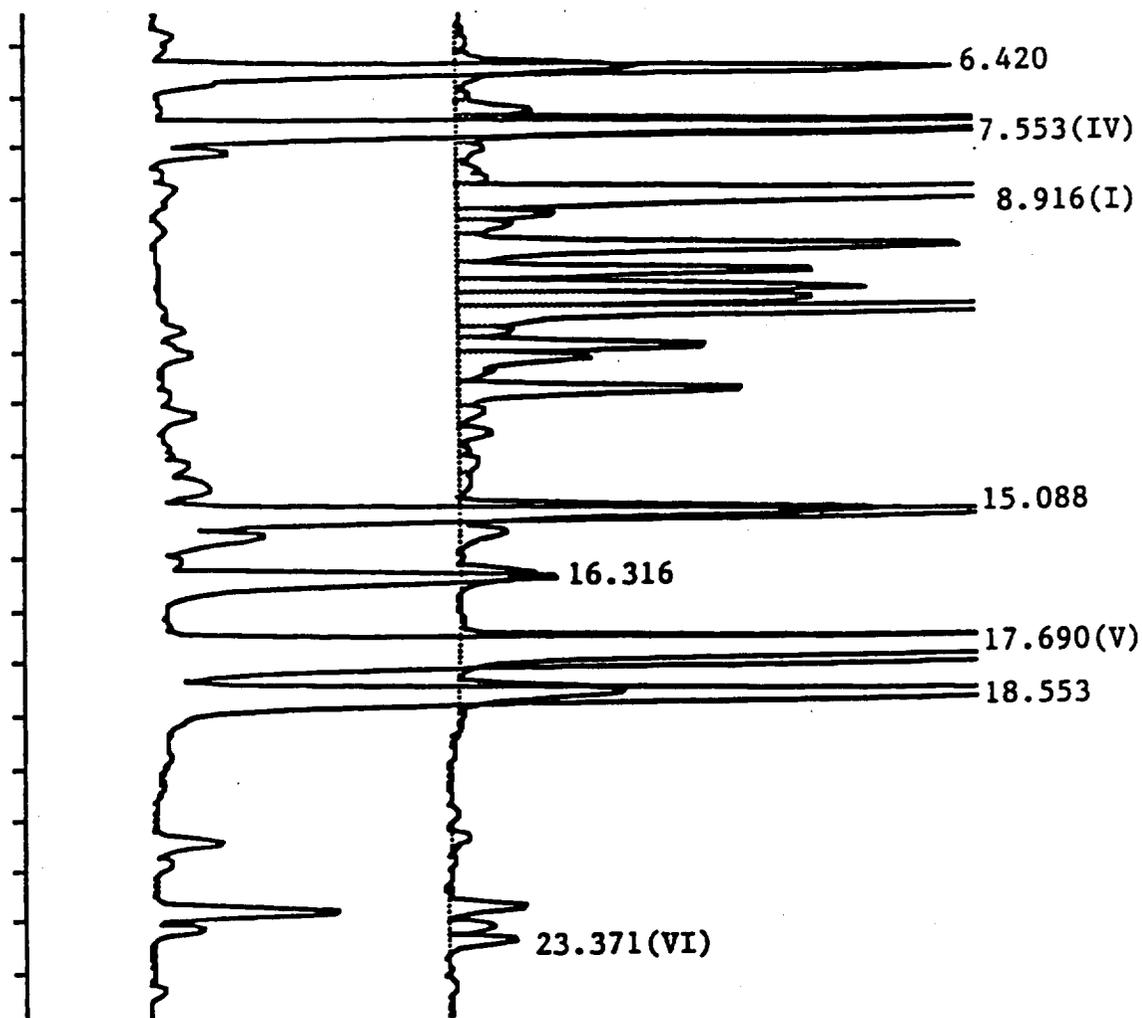


Figure 7. Chromatogram from extract of old coral sample from Station 2 (5/2/95); Hewlett-Packard HPLC system (see Results, section II, for full description of system). Numbers as described for Figure 5. Line to right indicates absorbance; line to left is fluorescence. Peak with RT of 15.088 is chl b; RT=16.316 is chl a allomer. Fluorescent peak preceding pigment IV is chlorophyllide a (RT=6.420). (See absorption spectra in Appendix C.)

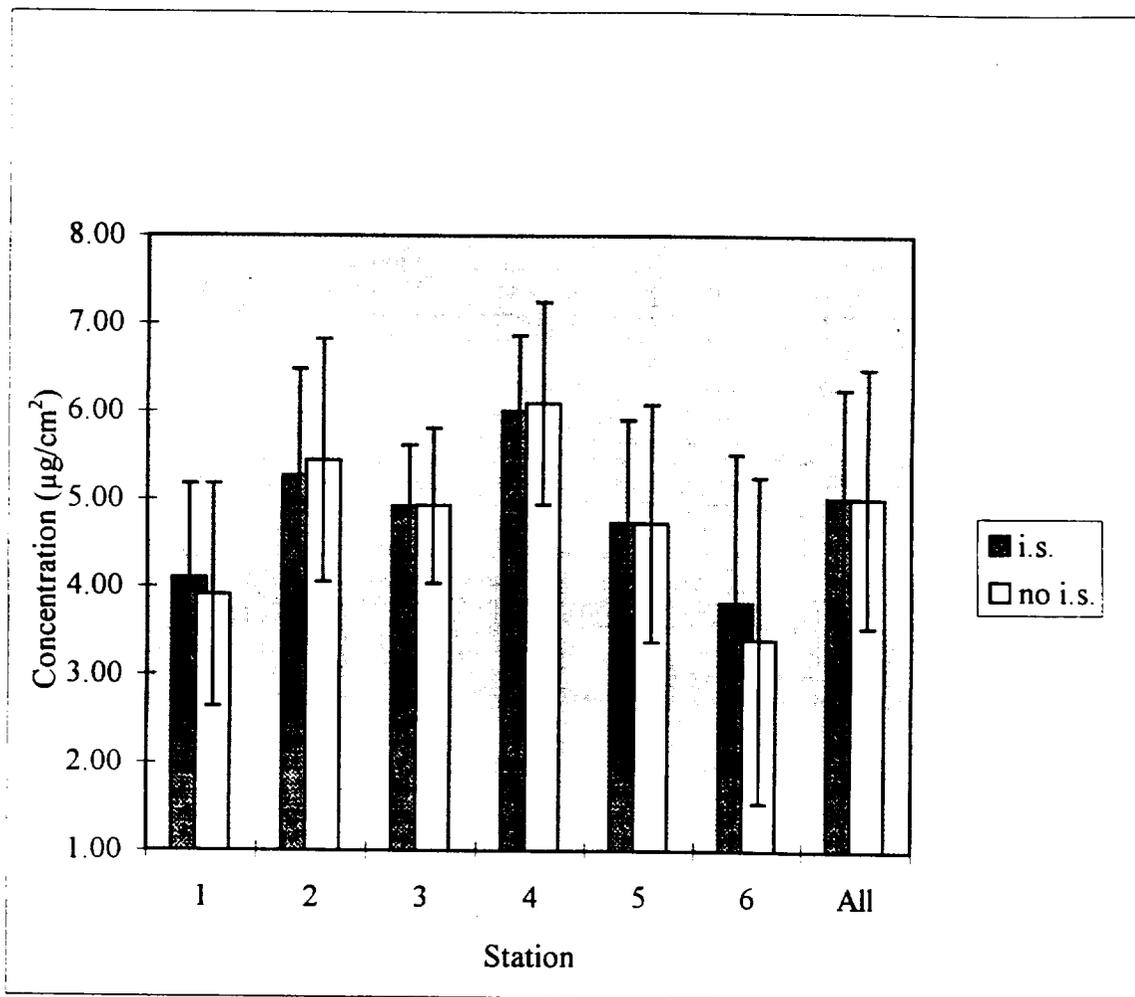


Figure 8. Mean peridinin concentrations, by station and for all stations, calculated with and without internal standard (i.s.) data. Error bars represent 1 SD. No significant differences were found between i.s. and no i.s. data for any station ( $\alpha=0.05$ ). Station 1,  $n=6$ ; Station 2,  $n=9$ ; Station 3,  $n=9$ ; Station 4,  $n=10$ ; Station 5,  $n=3$ ; Station 6,  $n=4$ ; All stations,  $n=41$ .

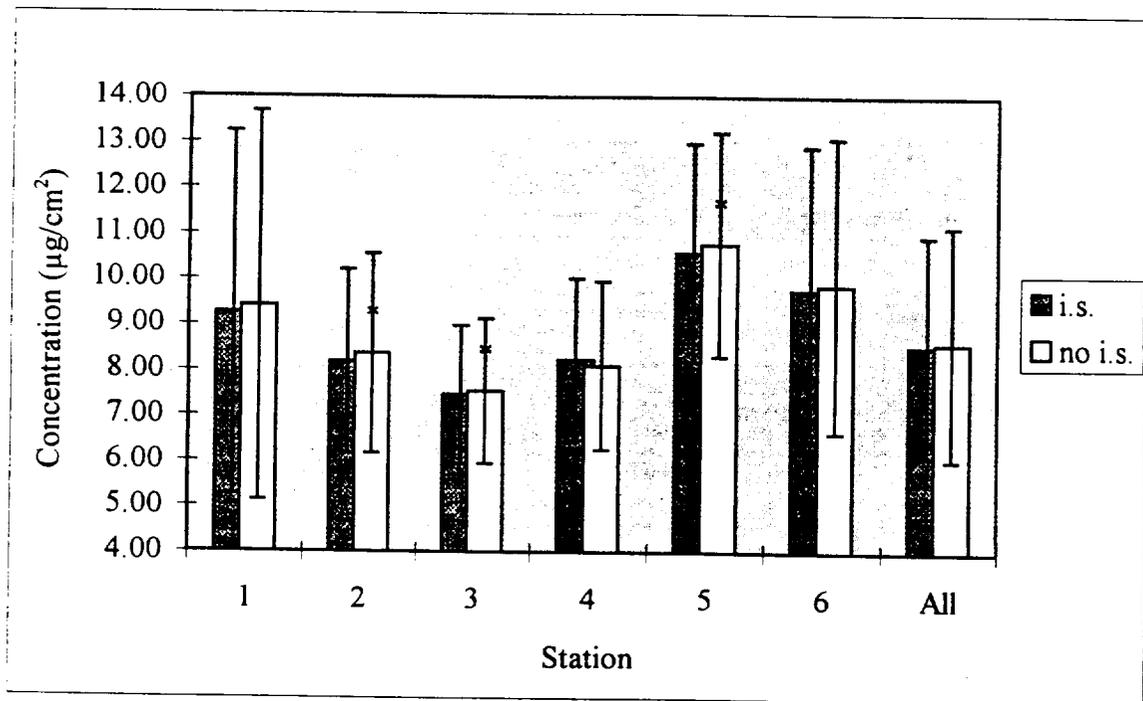


Figure 9. Mean chl a concentrations, by station and for all stations, calculated with and without i.s. data. Error bars represent 1 SD. "\*" indicates significant difference ( $p < 0.05$ ) between i.s. and no i.s. data. N values as described for Figure 8.

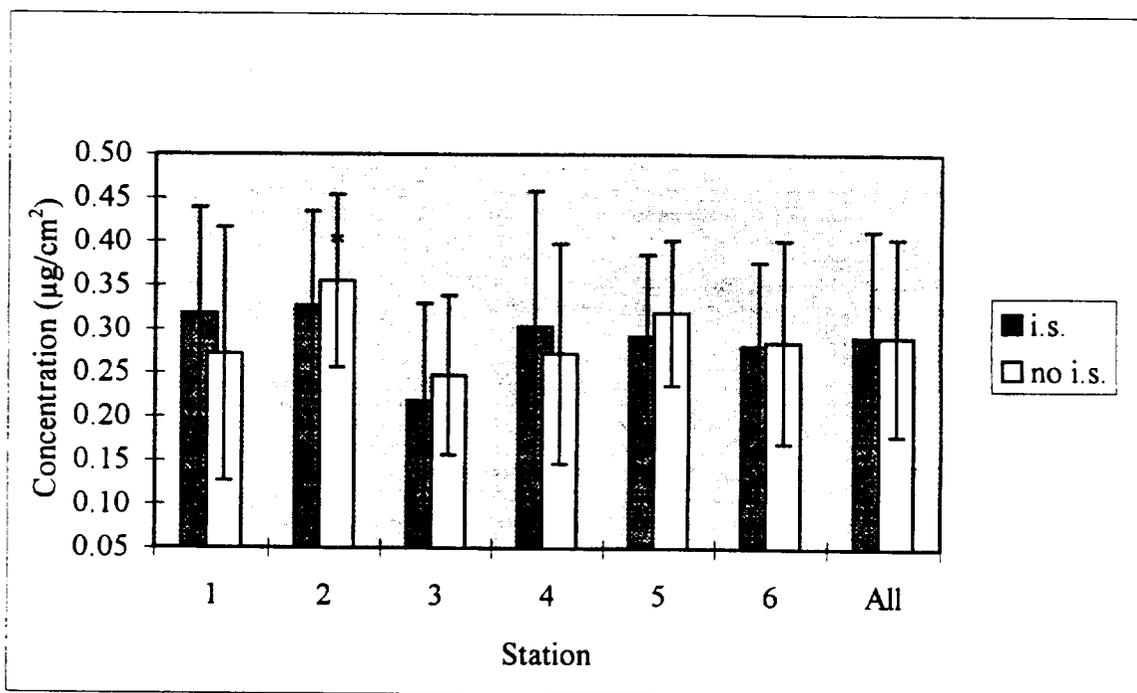


Figure 10. Mean  $\beta$ -car concentrations, by station and for all stations, calculated with and without i.s. data. Error bars represent 1 SD. "\*" indicates significant difference between i.s. and no i.s. data ( $p < 0.05$ ). N values as described for Figure 8, except: Station 3,  $n=8$ ; Station 4, no i.s.,  $n=9$ ; Station 6,  $n=3$ .

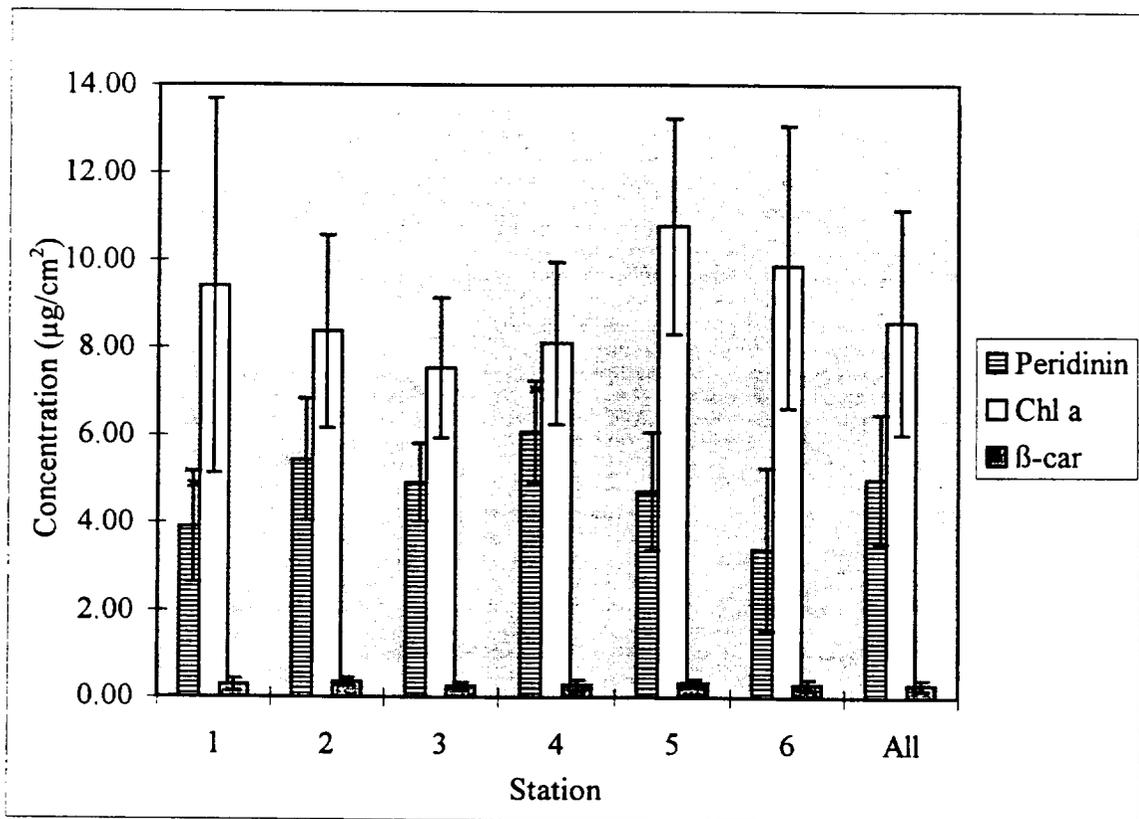


Figure 11. Mean pigment concentrations, by station and for all stations (calculated without i.s. data). Error bars represent 1 SD. "\*" signifies significant difference between stations, for pigment indicated. N values as described for Figures 8 and 10.

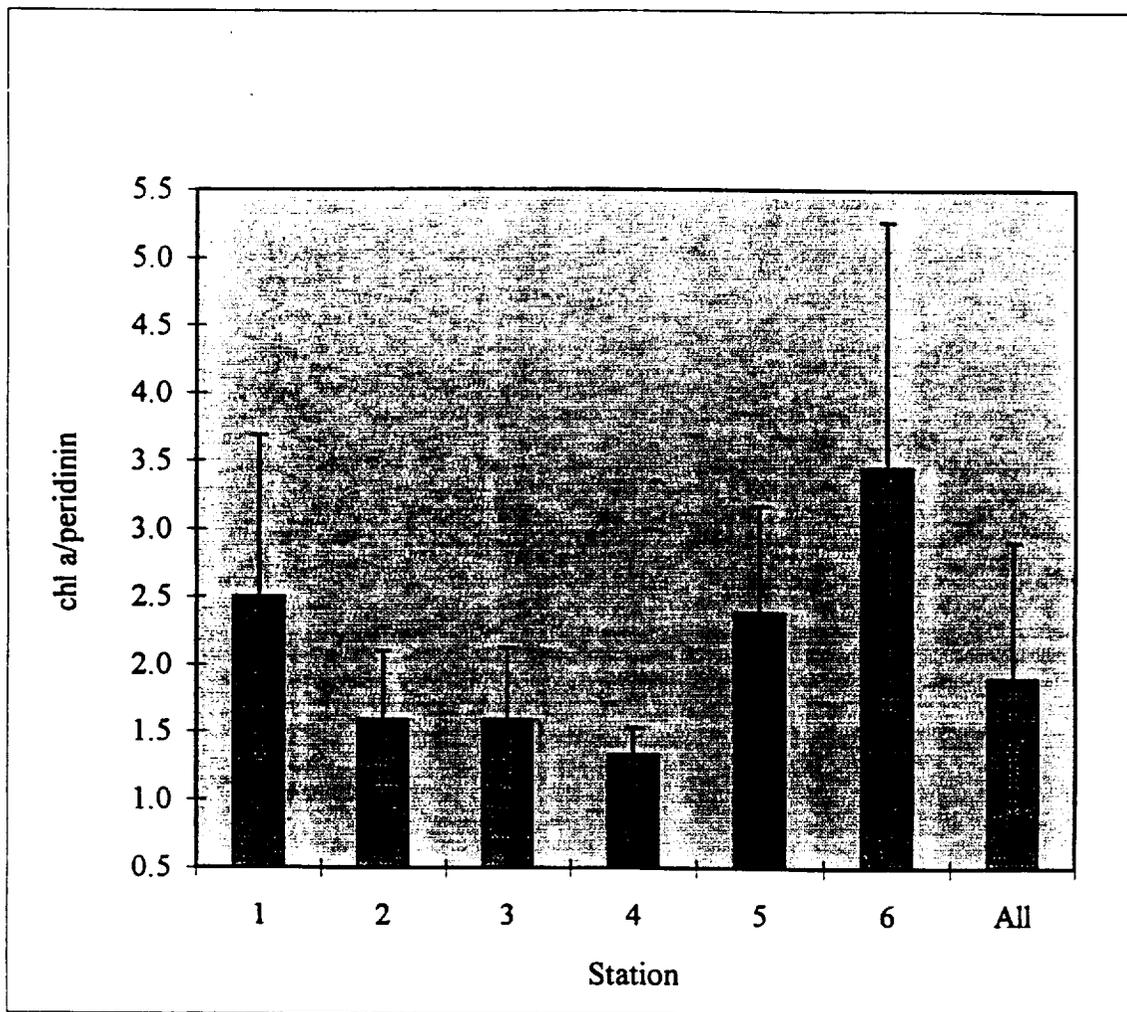


Figure 12. Mean chl a/peridinin concentration ratios, by station and for all stations (calculated without i.s. data). Error bars represent 1 SD. No significant pairwise differences were found between stations ( $\alpha=0.05$ ; see text). N values as described for Figure 8.

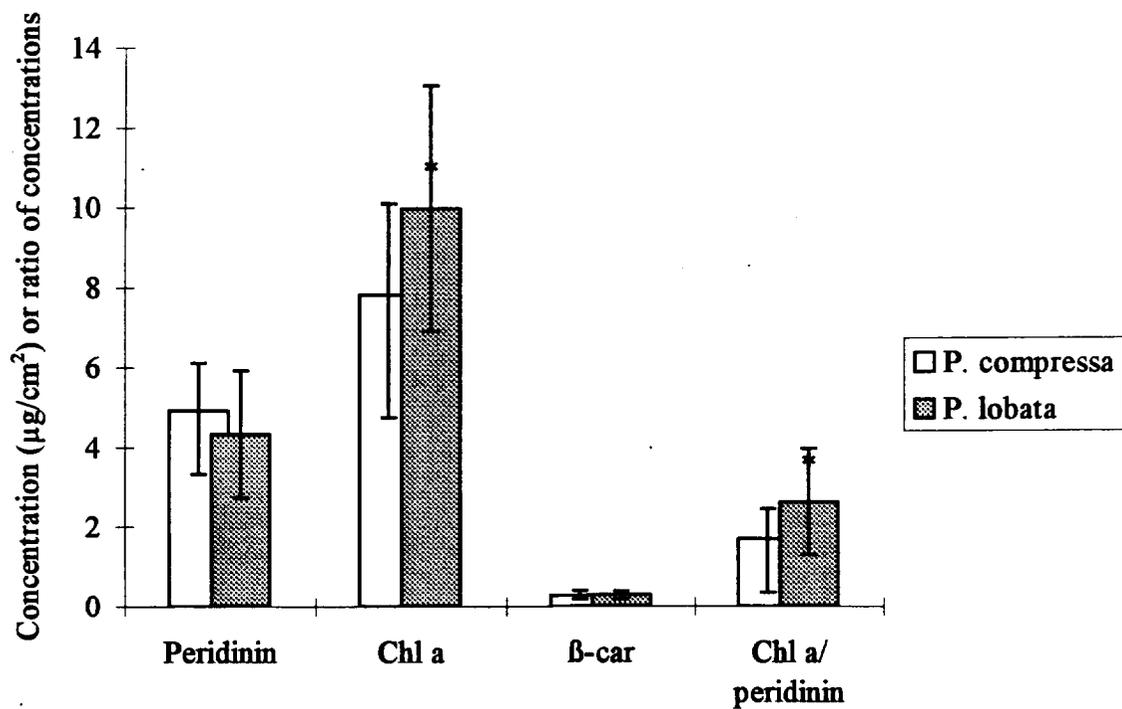


Figure 13. Mean coral pigment concentrations, and chl a/peridinin ratios, by species (calculated without i.s. data). Error bars represent 1 SD. "\*" indicates significant difference between species,  $p \leq 0.05$ . For *P. compressa*:  $n=25$  for peridinin, chl a, chl a/peridinin;  $n=23$  for β-car. For *P. lobata*:  $n=11$  for peridinin, chl a, chl a/peridinin;  $n=10$  for β-car.

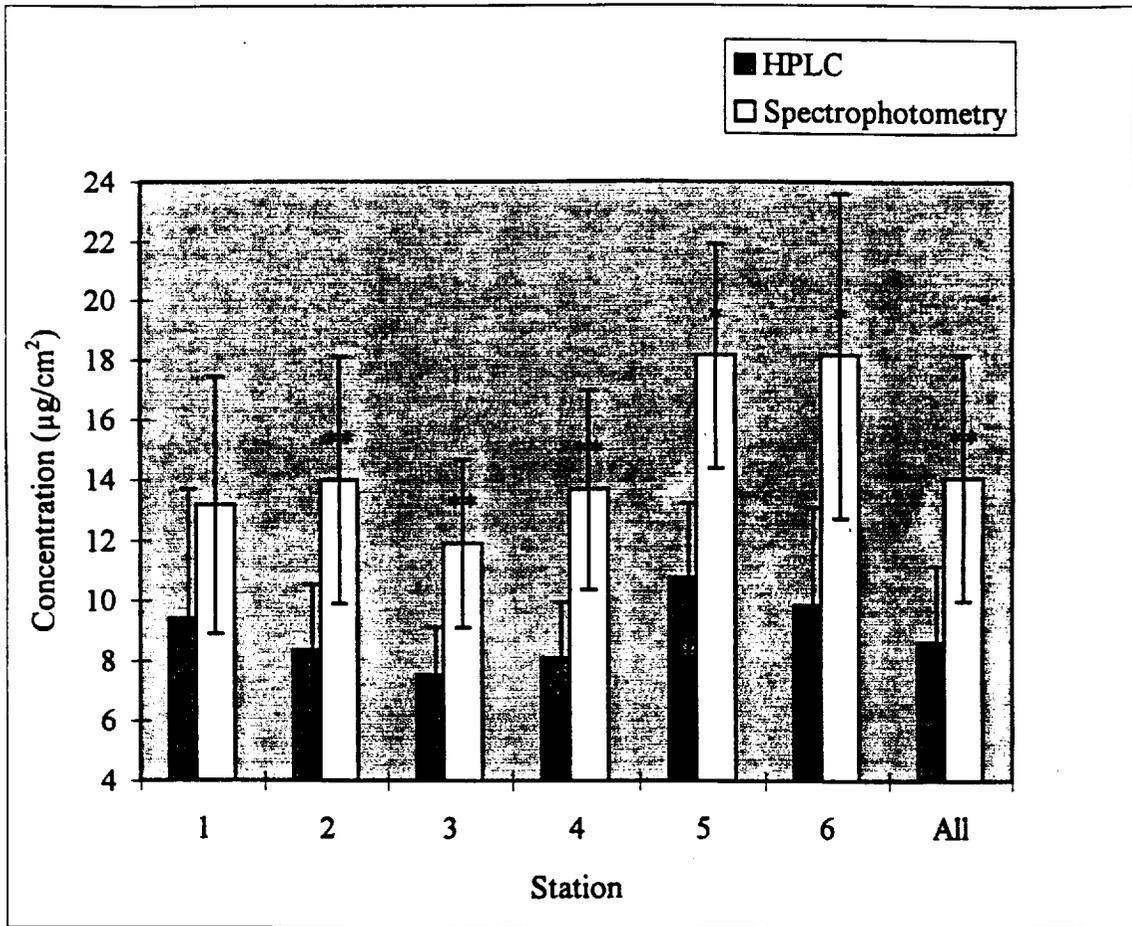


Figure 14. Mean chl a concentrations, determined by HPLC (no i.s.) and spectrophotometry (equations of Parsons et al. 1984), by station and for all stations. Error bars represent 1 SD. "\*" indicates significant difference between HPLC and spec. values,  $p \leq 0.05$ ; "\*\*\*" indicates highly significant difference,  $p < 0.001$ . No significant difference was found between spec. values by station ( $\alpha = 0.05$ ). N values as described for Figure 8.

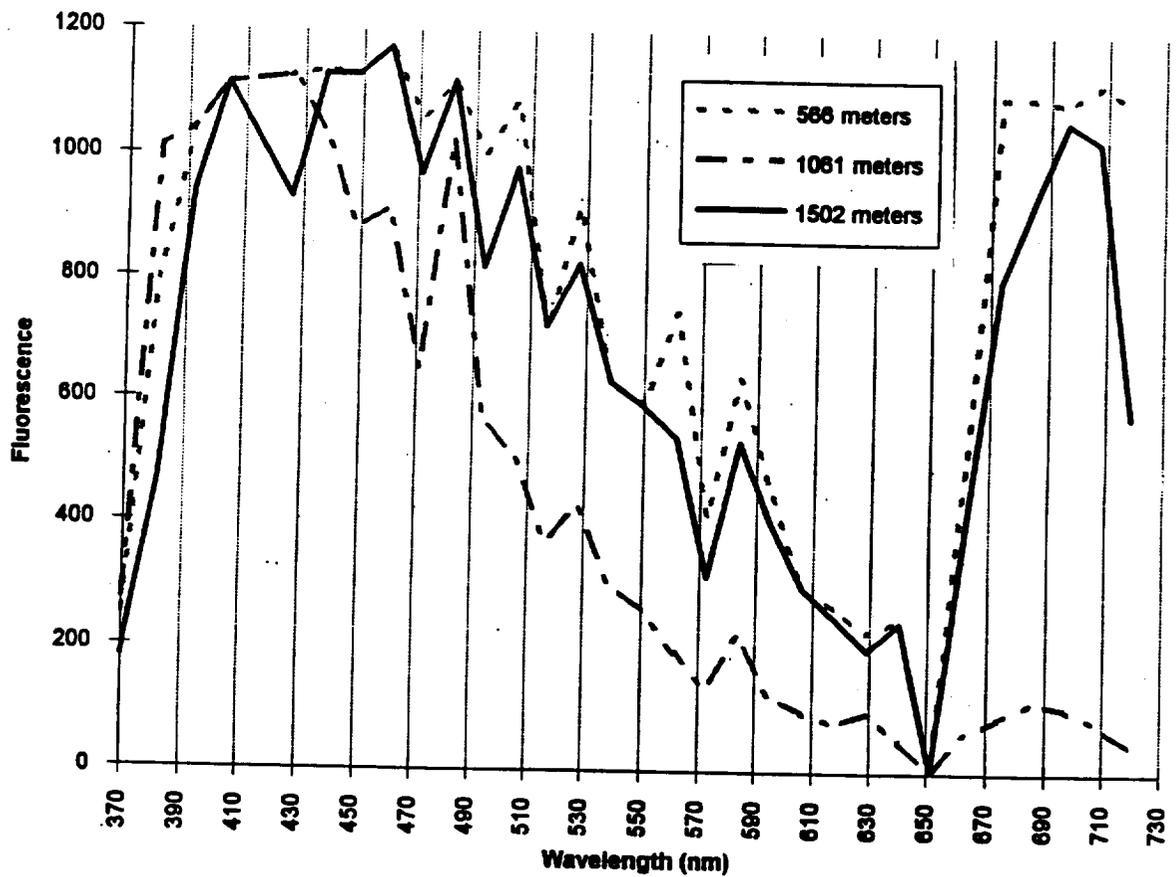


Figure 15. Three fluorescence spectra from reef at Puako, Hawai'i, generated using the NASA AOL system. Each spectrum corresponds to a different distance point along the aircraft transect (Transect 32222). Excitation wavelength = 355 nm (UV). Peaks around 685 nm represent chlorophyll fluorescence. Peaks below 600 nm may result from substances in coral tissue.

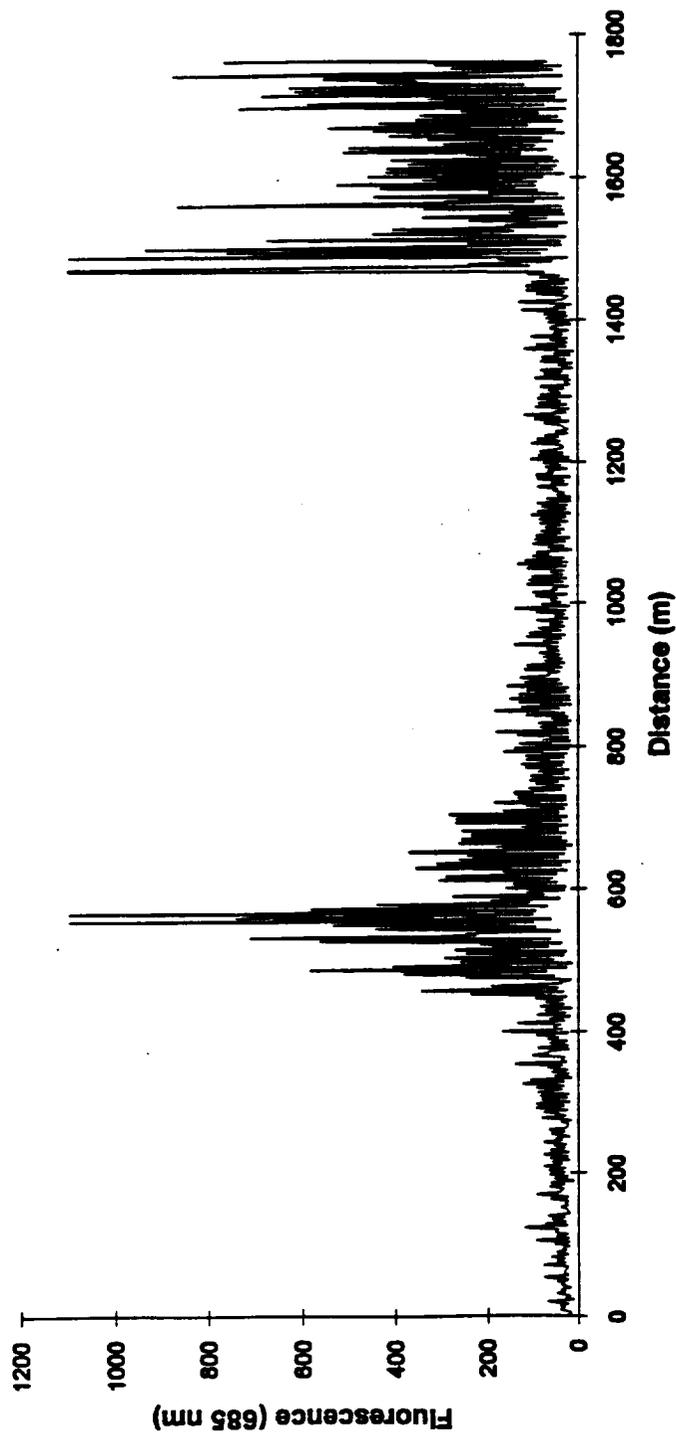


Figure 16. Chlorophyll fluorescence (685 nm) with distance along the reef transect at Puako, resulting from UV excitation (355 nm) using NASA AOL system. Depths ranged from <1 to >60 m.

<b>HPLC system</b>		
	<b>Waters</b>	<b>Hewlett-Packard</b>
<b>General description</b>	Waters gradient elution HPLC system: dual Waters Model 510 pumps; Waters automated gradient controller; Waters UK6 universal injector (manual injection); Spectra-Physics SP 4290 integrator	Hewlett-Packard Series 1050 HPLC: manual injection; Dynamax MacIntegrator II software system
<b>Used for</b>	Separation and collection of pigments from dinoflagellate extracts; tests of internal standards; calibration with pigment standards; quantification of pigments in coral samples	Identification of pigments in dinoflagellate and coral extracts
<b>Absorbance detector [detection wavelength]</b>	Waters Model 441 [436 nm]	Hewlett-Packard 1050 [436 nm]
<b>Fluorescence detector [excitation (Ex), emission (Em) wavelengths]</b>	-----	Waters 420 [Ex 350-500 nm (max 425 nm), Em >650 nm]
<b>Column(s)</b>	1) Waters C <sub>18</sub> ; 2) Spherisorb silica C <sub>18</sub> (pH stable) (both 5 µm particle size, 250 x 4.6 mm)*	Hewlett-Packard LiChrospher 100 RP-18 (5 µm particle size, 125 x 4 mm)
<b>Solvent system</b>	modified Mantoura and Llewellyn (1983); see Van Heukelem et al. (1992)*	see Wright et al. (1991)
<b>Gradient program</b>	see Mantoura and Llewellyn (1983)	see Wright et al. (1991)
<b>Flow rate (mL/min)</b>	1.00	1.00

Table 1. HPLC specifications, for the two systems used. \*See text (Methods, section F) for further explanation.

Station	Peridinin		Chl a, HPLC		Chl a, spec.		β-car		Chl a/ peridinin	
	mean	SD (%)	mean	SD (%)	mean	SD (%)	mean	SD (%)	mean	SD (%)
1	3.91	1.26 (32)	9.40	4.28 (46)	13.17	4.27 (32)	0.27	0.14 (52)	2.50	1.19 (48)
2	5.44	1.38 (25)	8.36	2.19 (26)	13.99	4.10 (29)	0.35	0.10 (29)	1.59	0.51 (32)
3	4.92	0.89 (18)	7.53	1.60 (21)	11.90	2.78 (23)	0.25	0.09 (36)	1.59	0.53 (33)
4	6.09	1.15 (19)	8.10	1.85 (23)	13.68	3.31 (24)	0.27	0.13 (48)	1.33	0.20 (15)
5	4.73	1.35 (29)	10.76	2.46 (23)	18.16	3.77 (21)	0.32	0.08 (25)	2.38	0.79 (33)
6	3.40	1.85 (54)	9.86	3.23 (33)	18.16	5.45 (30)	0.29	0.12 (41)	3.46	1.80 (52)
<b>All</b>	<b>5.01</b>	<b>1.47 (29)</b>	<b>8.59</b>	<b>2.56 (30)</b>	<b>14.05</b>	<b>4.09 (29)</b>	<b>0.29</b>	<b>0.11 (38)</b>	<b>1.90</b>	<b>1.00 (53)</b>

Table 2. Coral pigment concentration data, calculated without i.s. Mean concentration values are given in  $\mu\text{g}/\text{cm}^2$ , along with standard deviation (SD) and coefficient of variation (%). N values as described for Figures 8-10.

Pigment Concentrations ( $\mu\text{g}/\text{cm}^2$ )					
Reference	Technique	Notes	Peridinin	Chl a	$\beta$ -car
Gil-Turnes and Corredor (1981)	medium pressure solid-liquid chromatography	Values are: mean for specimens of <u>Porites astreoides</u> (range for 5 coral species). Numbers are estimates extrapolated from graph.	10.1 (4.5-14.2)	8.0 (4.4-14.3)	0.3 (0.1-0.4)
Kleppel et al. (1989)	HPLC	Values are: mean (SE), for "normal" (as opposed to "bleached") corals.	2.15 (0.30)	6.35 (1.05)	-----
Myers (1995)	HPLC (as described in this study)	Values are: mean for <u>Porites astreoides</u> specimens, mean for <u>P. porites</u> (range for 10 coral species)	3.5, 4.9 (0.4-4.9)	4.6, 4.8 (0.4-4.8)	-----
Cottone (this study)	HPLC (see Methods)	Values are: mean (SD), for all coral samples ( <u>Porites sp.</u> ).	5.01 (1.47)	8.59 (2.56)	0.29 (0.11)

Table 3. Summary of areal coral pigment concentration values reported in this and other studies.

## **APPENDIX A: Detailed procedures for extraction and quantification of coral pigments using HPLC**

### **1) Equipment and chemicals / handling of pigments**

All materials (glassware, plasticware, cuvettes, mortars and pestles, etc.) that were to come in contact with pigment standards and/or samples were thoroughly cleaned with phosphate-free soap, rinsed ten times with tap water, then 5 times with distilled water, then 3 times with nanopure water. They were acetone rinsed, then rinsed again with nanopure water. They were kept cold (at a maximum of 4°C; usually at -10°C immediately prior to use) and acid-free, to prevent pigment degradation.

Whenever possible, pigment standards and samples were handled in a nitrogen (oxygen-free) atmosphere, under dim red light, and were kept as cold as possible, to prevent pigment degradation. A glove box filled with nitrogen, with an attached fluorescent light covered with several layers of red cellophane, provided such an environment. On days when standards and/or samples were to be used, they were kept on ice (when in glove box) or in a -10 to -20°C freezer, wrapped in aluminum foil to protect them from light. On days when not in use, standards and samples were stored in 2-mL polypropylene microcentrifuge tubes wrapped in aluminum foil, in a -80°C freezer.

All solvents used were HPLC-grade, and all H<sub>2</sub>O was nanopure.

### **2) Surface area determination and pigment extraction from coral samples**

A frozen sample was removed from storage at -80°C, and its surface area was determined immediately using the aluminum foil method (Marsh 1970). (Aluminum foil was molded to the coral skeleton, to match the surface of the coral tissue; the foil was removed, rinsed with nanopure water, and allowed to dry; later, the foil was weighed; from the mass, the surface area was calculated using a standard curve of foil surface areas vs. masses)

The sample was then smashed and ground into a fine paste using a cast iron mortar and pestle. The homogenate was transferred to a 50-mL polypropylene centrifuge tube, using a spatula. The cast iron mortar and pestle were rinsed repeatedly with small amounts of 90% acetone to remove all traces of homogenate, and this rinse was added to the centrifuge tube (via a smaller, porcelain mortar, to prevent spillage). Enough 90% acetone was added to the centrifuge tube to adequately cover the homogenate. If the coral sample was small, the final volume of extract, after two extractions, was usually 25 mL; therefore, the volume of 90% acetone used for the first extraction did not exceed approximately 12 mL. If the sample was larger, the final volume of the extract was generally 50 mL, and the volume of 90% acetone used for the first extraction did not exceed 25 mL.

The centrifuge tube was capped and shaken thoroughly, wrapped in aluminum foil, and placed in a 0°C freezer overnight (10 to 14 hours) to extract the pigments. The centrifuge tube was shaken periodically, to facilitate extraction.

The procedures described above (surface area determination, grinding, pigment extraction) were repeated with 4 or 5 more coral samples on one day, so that a total of five or six samples were analyzed at one time.

The following day, the centrifuge tubes were shaken and centrifuged at 5000 RPM, at <0°C (in a refrigerated centrifuge), for 10 minutes. The supernatant from each sample was transferred into a separate volumetric flask (either 25 mL or 50 mL volume, depending on the size of the coral sample, as explained above), using Pasteur pipettes. (For several samples, the final volume of extract, after two extractions, was less than 25 mL or greater than 50 mL; in these cases, appropriately-sized [10-mL, or 100-mL] flasks were used. Ideally, all sample sizes and extraction volumes would have been the same.) The flasks were labelled, wrapped in foil, and stored at -15 to -20°C.

More 90% acetone was added to the centrifuge tubes from which the supernatants had been removed, to volumes of <12 mL for small samples or <25 mL for larger samples. (Care was taken not to exceed the volume of the flask containing the extract for each sample.) The centrifuge tubes were capped, shaken thoroughly, wrapped in aluminum foil, and extracted at 0°C for another 3-4 hours. The tubes were then centrifuged and the second volume of extract (supernatant) for each sample was added to the first extraction volume, in the appropriate volumetric flask, as described in the previous paragraph.

The volume of extract for each sample was made up to either 25 or 50 mL (as appropriate to the size of the volumetric flask used) with 90% acetone.

### 3) HPLC pigment analysis

#### a) Preparation of pigment standards

*i) Chlorophyll a and  $\beta$ -carotene:* Pure, crystalline chlorophyll (chl) a and  $\beta$ -carotene ( $\beta$ -car) were purchased from Sigma. One mg of pure chl a was dissolved in exactly 100 mL 90% aqueous acetone (using a volumetric flask), to make primary standard chl a. Approximately 1-3 mg of pure  $\beta$ -car were dissolved in exactly 100 mL ethanol, to make primary standard  $\beta$ -car. (Note: several "batches" of  $\beta$ -car primary standard were prepared between 3/93 and 7/94, due to the presence of undissolved "specks" of  $\beta$ -car in the "old" primary standard, which made the spectrophotometric absorbance readings fluctuate. However, specks were found in newly-prepared standard as well. Thus, the specks seemed to be due to a lack of solubility of the crystalline pigment in the ethanol, rather than the age of the standard. The specks were removed by centrifugation, as described in section bi, below.)

*ii) Peridinin and accessory pigments:* Coral pigment standards which could not be purchased were prepared from laboratory cultures of the dinoflagellates Heterocapsa pygmaea and Gymnodinium simplex, provided by Dr. Suzanne Strom of Shannon Point Marine Center, Anacortes, WA. The cultures were vacuum-

filtered through 47-mm diameter Whatman GF/C filters and washed with culture medium (f/2) or autoclaved, filtered (0.2  $\mu\text{m}$ ) seawater. The filters were ground, using a mortar and pestle, in 2-3 mLs of cold 90% aqueous acetone, for 30-60 seconds. The homogenate was transferred into 13-mL graduated centrifuge tubes, and the volume of each tube was made up to between 7 and 13 mL with 90% acetone (from repeated washing of the mortar and pestle with small volumes of acetone, to remove all pigment). The centrifuge tubes were shaken, wrapped in aluminum foil and placed in a refrigerator (4°C) overnight (14-16 hours), to extract the pigments.

The centrifuge tubes were shaken thoroughly, then centrifuged (5000 RPM) for 10-15 minutes. The supernatants were decanted into a cold graduated cylinder. (Extracts from the same genus of dinoflagellate were combined in one graduated cylinder; Heterocapsa and Gymnodinium extracts were kept separate.)

When a significant amount of pigment remained in the ground filters after the first extraction, a second extraction was performed. More 90% acetone was added to the centrifuge tubes containing the ground filters; the filters were extracted overnight, and the supernatant was treated similarly to that from the first extraction.

The dinoflagellate extracts were then concentrated, using Sep-Pak C<sub>18</sub> cartridges. The extracts, in graduated cylinders, were diluted with nanopure water to double their volume. The diluted extracts were loaded onto Sep-Pak C<sub>18</sub> cartridges (which had been conditioned with methanol and water) and eluted in 100% acetone. The elution volume was approximately 1/5 of the volume loaded onto the column (e.g., 25 mL of diluted extract were concentrated to 5 mL of extract in pure acetone).

The major pigments of the concentrated dinoflagellate extracts were separated and collected using a Waters HPLC system (see Methods, section IIF). First, stable chromatographic conditions were established, and a blank of acetone+water (ratio of 2:1) was injected (volume=80  $\mu\text{L}$ ), to check for any contamination of the system.

(Contamination could produce visible peaks on the chromatogram, which could interfere with the pigment peaks of interest.) Next, a small volume (e.g., 1000  $\mu$ L) of extract was diluted by 50% (e.g., 500  $\mu$ L) with water. The extract+water was mixed thoroughly, and 360–450  $\mu$ L were injected into the chromatograph. (In initial trials, the extract was filtered [Gelman FP-450 Vericel Membrane Filters, 13 mm diameter, 0.45  $\mu$ m pore size] or microcentrifuged at high speed prior to HPLC injection. However, this clarification step was abandoned; the extract proved to be clear enough for HPLC injection after passing through the Sep-Pak cartridge.)

The pigments corresponding to the major peaks on the chromatograms were collected in separate microcentrifuge tubes as they eluted. The absorbance detector signaled the elution of each pigment from the column; pigments were collected at the tops of the chromatogram peaks, to avoid cross-contamination.

360–450  $\mu$ L volumes of extract+water were injected repeatedly, and the separated pigments were collected, until an adequate volume of each pigment had been collected for spectrophotometric identification (see below) and (in the case of peridinin) use as a primary standard.

Each of the separated pigments was concentrated and transferred into a pure solvent (from the mixed eluent in which it eluted), using a Sep-Pak cartridge, as follows: All of the pigment corresponding to one of the major peaks was combined in a cold graduated cylinder. The pigment was diluted with cold water to double its volume, and the diluted pigment was loaded onto a Sep-Pak cartridge. The pigment was eluted in 90% acetone (chl a), methanol (peridinin), or ethanol ( $\beta$ -car, diadinoxanthin, dinoxanthin), depending on the solvent appropriate for the literature extinction coefficient and absorbance values for the suspected pigment (Strain et al. 1944; Taylor 1967; Jeffrey 1968; Jeffrey and Haxo 1968; Mantoura and Llewellyn 1983). The pigment was eluted using just enough pure solvent to remove all pigment from the column. When a relatively

large volume of a pigment had been collected, (e.g., peridinin and chl a), a standard 1-cm cuvette (VWR, optical glass) was used for subsequent spectrophotometric identification (see below), and a volume of about 4 mL of visibly-colored pigment was needed. When only a small amount of a particular pigment had been collected (i.e., the smaller peaks on the chromatograms, such as  $\beta$ -car, dinoxanthin and diadinoxanthin), a small-volume, 5-cm-path-length cuvette (Starna, Micro Short glass cell) was used for spectrophotometry, and a volume of approximately 2 mL of faintly-colored pigment was needed.

An absorption spectrum (800-315nm) was obtained for each purified pigment (Perkin-Elmer Lambda 3B UV/VIS spectrophotometer, with Perkin-Elmer 3600 Data Station, PETOS operating system, IFL3 software). The purified pigments were identified by comparing their spectra to published absorption spectra and absorption maxima (Strain et al. 1944; Taylor 1967; Jeffrey 1968; Jeffrey and Haxo 1968; Mantoura and Llewellyn 1983), and by comparing their HPLC chromatograms to published chromatograms of algal extracts (Mantoura and Llewellyn 1983; Wright and Shearer 1984; Kleppel et al. 1988; Wright et al. 1991). Unpublished pigment spectra and dinoflagellate chromatograms, provided by S. Strom, also aided in the identification of the pigments. In addition, algal extracts were "spiked" with pure, prepared standards of chl a and  $\beta$ -car, and injected into the HPLC; the suspected chl a and  $\beta$ -car peaks on the algal chromatograms were confirmed when these peaks were increased in size by the spike. Finally, the dinoflagellate extracts were run on a Hewlett-Packard HPLC system which included a fluorescence detector (see Table 1 for details of instrumentation). The chlorophyll and chlorophyllide pigments were easily identified by their fluorescence.

*iii) Internal standards, trans- $\beta$ -apo-8'-carotenal and ethyl  $\beta$ -apo-8'-carotenoate:* Pure  $\beta$ -apo-8'-carotenal ( $\beta$ -apo) and ethyl  $\beta$ -apo-8'-carotenoate (ethyl  $\beta$ ) were purchased from Fluka. Approximately 9 mg of  $\beta$ -apo were dissolved in exactly 100

mL ethanol (in a volumetric flask), to make the primary standard. The primary standard for ethyl  $\beta$  was prepared similarly.

#### **b) Calibration / Standardization**

*i) Spectrophotometric determination of pigment standard concentrations:* Prior to HPLC injection of standards, the exact concentrations of the primary standards were determined spectrophotometrically. After the Perkin-Elmer Lambda 3B UV/VIS spectrophotometer had been "zeroed" with two 90% acetone blanks, 3-4 mL of chl a primary standard were transferred into a cuvette (VWR optical glass, 1 cm path length) and absorbance was measured at 440, 664, and 750 nm. The concentration of the solution was calculated using Beer's law, equations from Davies (1976), and extinction coefficients given by Mantoura and Llewellyn (1983), as described in Appendix B. The concentrations of the  $\beta$ -car and peridinin primary standards were determined similarly; absorbance was measured at 440 and 750 nm.

For greatest accuracy, absorbance values should fall between 0.200 and 0.800. Since the absorbances (at 440 nm) measured for the chl a and peridinin primary standards fell within the acceptable range, no dilution or concentration of the standards was necessary. In some cases (i.e., the second "batch" of standard), the  $\beta$ -car primary standard was diluted by 50% with ethanol, to reduce its absorbance value. The  $\beta$ -car primary standard also required centrifugation (15000 RPM, for 10 min) before stable absorbance readings could be obtained, due to the presence of undissolved bits of pigment (see section ai, above).

The internal standard primary standards required quantitative dilution. The absorbance of the  $\beta$ -apo primary standard was adjusted to approximately 0.500 by diluting 1 part standard with 31 parts ethanol. The ethyl  $\beta$  primary standard was diluted 1:7, standard:ethanol to obtain an absorbance value of approximately 0.500.

*ii) Calibration curves:* A calibration curve was constructed for each pigment standard, by plotting the mass of pigment injected into the chromatograph vs. the area under the resultant pigment peak on the chromatogram.

Once stable chromatographic conditions had been established, a solvent blank was injected (80  $\mu$ L) as described in section 3aii. (The solvent injected was the same solvent in which the pigment of interest was dissolved.) Following spectrophotometry (see previous section), the approximately 3-4 mL of each primary standard which had been used in spectrophotometry were returned to microcentrifuge tubes and centrifuged (15000 RPM,  $< 0^{\circ}$ C) for 5 min. (In the case of the internal standards, the quantitatively-diluted standards, which had been used in spectrophotometry, were centrifuged in preparation for HPLC injection, rather than the original, concentrated primary standards.)

At least 5 working standards, of various concentrations, were prepared from each primary standard of known concentration, one at a time, and injected. Increasingly dilute working standards were prepared from the primary standard by increasing the ratio of solvent to standard, while keeping the total volume of the mixture constant (e.g., working standard #1 = 400  $\mu$ L  $\beta$ -apo primary standard (ps); #2 = 300  $\mu$ L  $\beta$ -apo ps + 100  $\mu$ L ethanol; #3 = 200  $\mu$ L  $\beta$ -apo ps + 200  $\mu$ L ethanol . . .). Each working standard was diluted with water (2:1, standard:water) and mixed thoroughly before 80  $\mu$ L were injected. Care was taken to ensure that the concentrations of the working standards spanned the range of pigment concentrations expected in the actual samples to be analyzed. (Approximations were made based on trial HPLC injections of coral extract, and from the relative peak heights on the dinoflagellate chromatograms discussed previously.)

The amounts of each pigment injected were calculated (from the known concentration of the primary standard and the amount of standard injected; see Appendix

B) and plotted against the areas under the corresponding peaks on the chromatograms, to produce a calibration curve for each pigment (Figure A1).

Once the calibration curves for the individual pigments were found to be linear and pass through the origin, a series of calibration mixtures of standards was prepared and injected. Calibration curves were then created, as follows, in which each pigment was considered in ratio to the internal standard (in most cases,  $\beta$ -apo), rather than individually.

At least 5 mixtures of primary standards, of known concentrations, were prepared and injected into the chromatograph. All mixtures contained the same amount of internal standard (usually  $\beta$ -apo, quantitatively-diluted, as described in section bi) and different proportions of the other standards. For example: Mixture A=100  $\mu$ L  $\beta$ -apo+200  $\mu$ L peridinin+200  $\mu$ L chl a+100  $\mu$ L  $\beta$ -car; Mixture B=100  $\mu$ L  $\beta$ -apo+150  $\mu$ L peridinin+300  $\mu$ L chl a+50  $\mu$ L  $\beta$ -car . . . Each mixture was diluted 2:1, mixture:water, and mixed thoroughly, before 80  $\mu$ L were injected (Figure A2).

As in the creation of the original calibration curves (see above), the pigment concentrations in the calibration mixtures were made to cover the range of concentrations expected to be found in the samples analyzed. Table A1 describes the (approximate) spectrophotometric absorbance values (at 440 nm) for each pigment standard, which resulted in peak areas appropriate for the peak areas found on the coral sample chromatograms, when 80  $\mu$ L of each of the mixtures described above were injected.

A calibration curve was prepared for each pigment of the mixture, in which Area (under peak on chromatogram)<sub>pigment</sub>/Area internal standard (i.s.) was plotted against  $\mu$ g injected<sub>pigment</sub>/ $\mu$ g injected<sub>i.s.</sub> (Figure A3; see Appendix B for calculations of " $\mu$ g injected;" Johnson and Stevenson 1978)

*iii) Daily calibration:* For "daily" calibration (i.e., calibration on the day that samples were to be analyzed) only 2 or 3 calibration mixtures of standards

needed be used, to redefine the slope of each curve created from the standard mixtures injected previously (section bii; Kaiser and Debbrecht 1977).

The concentrations of all standards were determined spectrophotometrically, as described above (section bi), either the day before or the day of HPLC analysis of samples. (Concentrations had to be determined prior to each round of calibrations and HPLC sample runs, since even slight solvent evaporation over time changed the pigment concentrations of the primary standards.) The pigment standards that had been used in spectrophotometry were then centrifuged at high speed, as described above.

On the day of HPLC injection of sample extracts, 2-3 mixtures of primary standards, of known concentrations, were prepared and injected into the chromatograph, as described in section bii. Generally, each of the two calibration mixtures described previously was injected once prior to injection of sample extracts, and one of the two mixtures was injected again at the end of the day, once all of the samples had been run (in case chromatographic conditions had changed significantly during the course of the day).

A calibration curve was prepared for each pigment of the mixture, plotting ratios of pigment to i.s., as described in bii. If the data points from the daily calibration mixtures "fit" the previously-generated calibration curve, the data from the 2 or 3 daily calibration injections of standard mixtures were added to the calibration curves prepared previously, and the equations for the previous regression lines were recalculated (Grob 1977). If the daily calibration data points did not fit the curve, a new regression equation was calculated based on the new data points. A new regression equation was calculated for each pigment every day that samples were analyzed and new calibration data points were added.

### **c) Sample analysis**

Once HPLC calibration with pure standards of peridinin, chl a, and  $\beta$ -car had been completed, the pigments were extracted from the coral samples, as described in section 2, and were injected for HPLC quantification.

While the samples were undergoing their second extraction (see section 2), stable chromatographic conditions were established and a solvent (ethanol or 90% acetone) blank was injected. Then, two calibration mixtures were injected, as described in section biii.

Immediately following the second extraction of each sample, once the volume in each volumetric flask had been brought up to the mark with 90% acetone (see section 2), the same relative amount of concentrated internal standard (i.e., the original primary standard, before quantitative dilution for spectrophotometry) was added to each flask; i.e., 200  $\mu\text{L}$  of  $\beta$ -apo were added to each 50 mL flask, and 100  $\mu\text{L}$  were added to each 25 mL flask. (The concentration of the concentrated i.s. was determined by multiplying the concentration of the quantitatively-diluted standard by the dilution factor. For example,  $[\beta\text{-apo}]_{\text{conc}} = 32[\beta\text{-apo}]_{\text{diluted}}$ .)

The contents of each flask were mixed thoroughly. Approximately 10 mL from each flask were transferred into a polypropylene centrifuge tube, and the tubes were centrifuged at 15000 RPM, at  $<0^{\circ}\text{C}$ , for 5 minutes. Approximately 4 mL of extract from each tube were removed for spectrophotometric analysis (see Methods section of text).

A small volume of centrifuged sample extract was then diluted 2:1, sample:water (e.g., 400  $\mu\text{L}$  sample extract + 200  $\mu\text{L}$  water), and 80  $\mu\text{L}$  of the diluted extract were injected into the chromatograph. The dilution and injection procedure was repeated for each sample extract. After 80  $\mu\text{L}$  of each sample extract had been injected once, another calibration mixture was injected, as described in section biii.

The peaks corresponding to peridinin, chl a,  $\beta$ -car, and the i.s. ( $\beta$ -apo, usually) on the sample chromatograms were identified. The ratios of the areas under the peridinin, chl a, and  $\beta$ -car peaks to the areas under the i.s. peak were calculated, for each sample. The calibration curves created from the calibration mixtures, and the calibration curve created for the i.s., were then used to determine the amount ( $\mu\text{g}$ ) of each pigment injected, for

each sample (see Appendix B). The amount of each pigment injected was divided by the surface area of the coral sample (see section 2) to give the concentrations of peridinin, chl a, and  $\beta$ -car in each coral sample, in  $\mu\text{g}/\text{cm}^2$ .

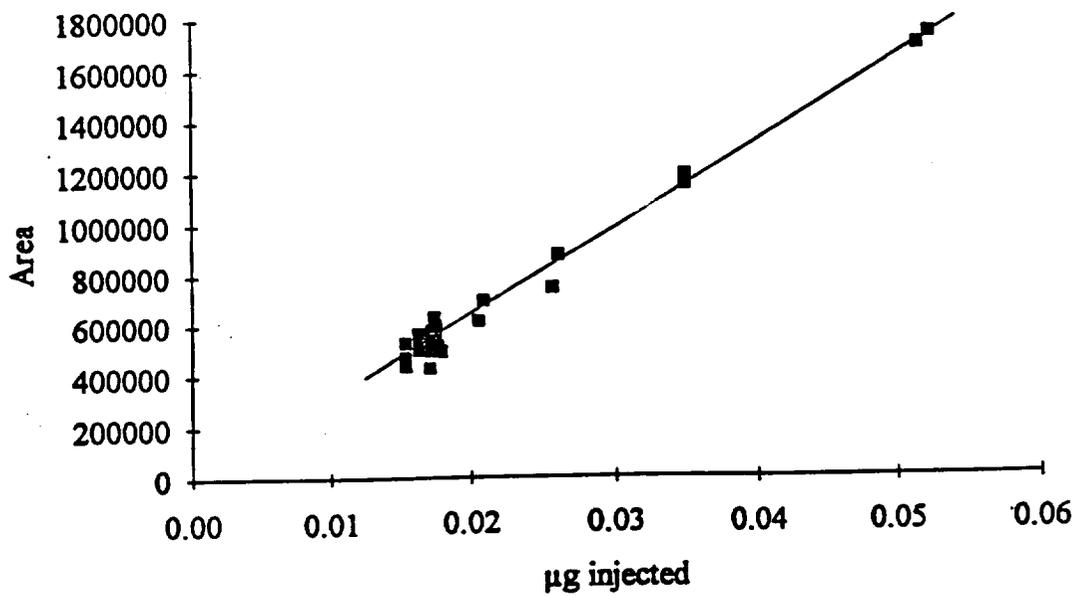


Figure A1. Example of a calibration curve for  $\beta$ -apo-8'-carotenal, 8/3/94 (using data since 3/30/94). Equation for regression line:  $y = 33864503 x - 43673$  ( $r^2 = 0.98$ ,  $n=43$ ).

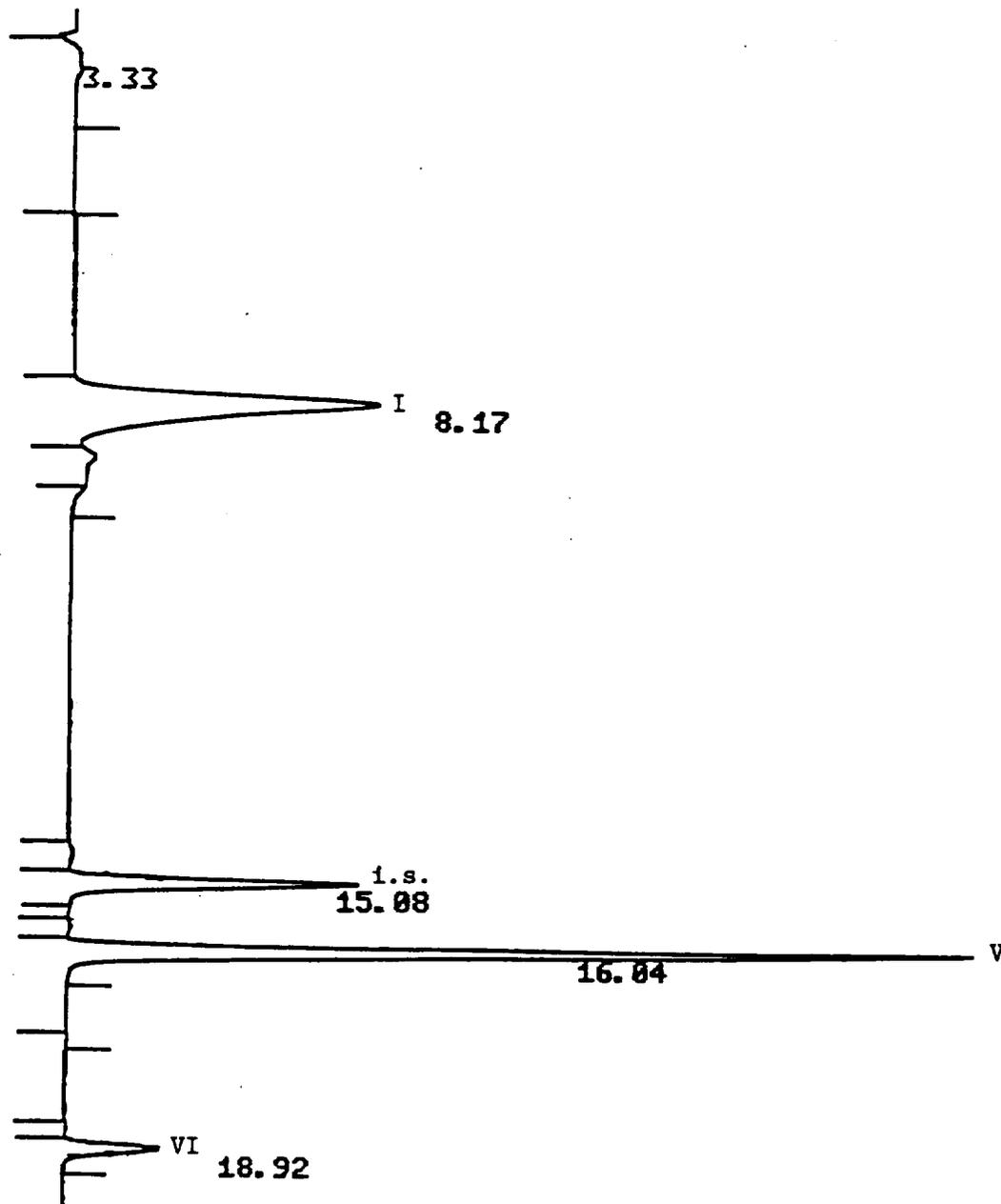


Figure A2. Chromatogram from calibration mixture: 100  $\mu$ L  $\beta$ -apo (i.s.) + 200  $\mu$ L peridinin + 200  $\mu$ L chl a + 100  $\mu$ L  $\beta$ -car + 300  $\mu$ L water, 80  $\mu$ L injected. Numbers as described for Figure 2. Waters HPLC system, Spherisorb column.

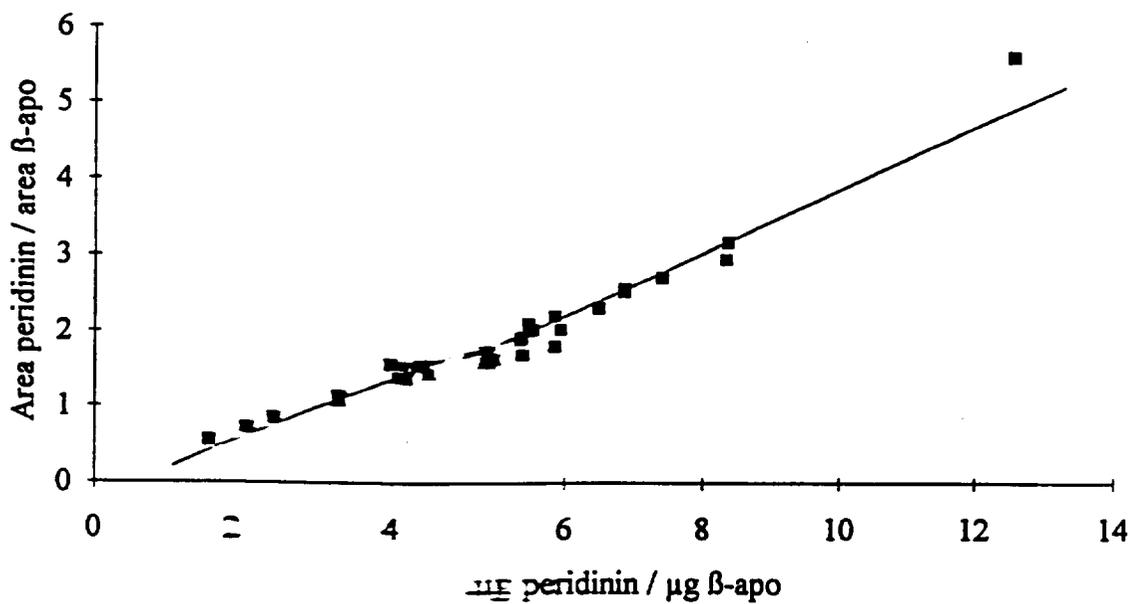


Figure A3. Example of a calibration curve for peridinin (8/3/94), in which the mass and area values for the pigment of interest are plotted in ratio to those of the i.s. ( $\beta$ -apo). Equation for regression line:  $y = 0.43x - 0.38$  ( $r^2 = 0.96$ ,  $n=33$ ).

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