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The presence of chlorophyll d in red algae

Mariá Elia Lanraú
San Jose State University

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THE PRESENCE OF CHLOROPHYLL *d* IN RED ALGAE

A Thesis

Presented to

The Faculty of the Department of Biology

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

María Elia Landráu

December 2000

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APPROVED FOR THE DEPARTMENT OF BIOLOGY

Dr. Nicholas A. Welschmeyer, Moss Landing Marine Laboratories

Dr. Michael S. Foster, Moss Landing Marine Laboratories

Dr. Chris Brinegar, San Jose State University

APPROVED FOR THE UNIVERSITY

ABSTRACT

THE PRESENCE OF CHLOROPHYLL *d* IN RED ALGAE

by María E. Landráu

Chlorophyll *d* has been cited as a chemotaxonomically unique pigment of red macroalgae. However, considerable uncertainty is associated with presence of chlorophyll *d* in red algae because 1) older, insensitive methods have often failed to detect chlorophyll *d*, and 2) numerous studies have suggested that chlorophyll *d* is simply an oxidation product of chlorophyll *a*, resulting as an artifact of extraction procedures. In this study, chlorophyll *d* was detected in 47 out of 57 species of red macroalgae from central California, one out of three species from Baja California Sur, Mexico, and one out of two species from Cape Evans, Antarctica. Chlorophyll *d* was not produced as an artifact of the routine extraction procedure and appears to be a natural component of red macroalgae. Chlorophyll *d* also was found in high enough concentrations to impart a detectable *in vivo* absorption band in the red (718 nm) spectral region.

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INTRODUCTION

Various species of red macroalgae have been reported to contain a second green pigment known as chlorophyll *d*, in addition to chlorophyll *a* (Manning and Strain 1943, Strain 1958, Holt 1961, Allen 1966, O'h Eocha 1971, Rowan 1989). The presence of chlorophyll *d* in red algae has been controversial because evidence for its existence has been inconsistent. It has been suggested that chlorophyll *d* is simply an artifact of the pigment extraction process because one of the oxidation derivatives of chlorophyll *a* has an absorption spectrum identical to that of chlorophyll *d* (Holt and Morley 1959, Holt 1961). Additionally, modern techniques that measure absorption spectra of chlorophylls *in vivo* provide no direct evidence for the presence of chlorophyll *d* in living cells of red algae (Rowan 1989).

Chlorophyll *d* was first isolated and described by Manning and Strain (1943) based on the analysis of 18 species of red algae collected near Moss Beach, north of Half Moon Bay, California, and analyzed. Manning and Strain (1943) observed a distinct shoulder around 700 nm in the absorption spectrum of crude methanol extracts and concluded that this shoulder indicated the presence of another chlorophyll-like pigment, other than chlorophyll *a*; they named it chlorophyll *d*. Manning and Strain (1943) reported that purified chlorophyll *d* (in methanol) had an absorption maximum at 696 nm, a red shift of approximately 30 nm from the chlorophyll *a* absorption maximum at 665 nm. The fluorescence excitation and emission maxima of chlorophyll *d* in diethyl ether were reported at 445 nm and 693 nm, respectively (Manning and Strain 1943).

In the early study of Manning and Strain (1943), the authors tested various extraction procedures with *Erythrophyllum delesserioides*, the species that yielded the most chlorophyll *d*, to determine if the presence of chlorophyll *d* was a result of their extraction method. Results showed the proportion of chlorophyll *d* relative to chlorophyll *a* did not change with variation in extraction procedure, suggesting chlorophyll *d* occurs naturally.

However, it was later proposed that chlorophyll *d* could be formed synthetically as an oxidation product of chlorophyll *a* (Sagromsky 1958, Holt and Morley 1959). The chemical structure of chlorophyll *d* was proposed by Holt and Morley (1959) to be identical to 2-desvinyl-2-formyl chlorophyll *a* (3-desvinyl-3-formyl chlorophyll *a* by current nomenclature under International Union of Pure and Applied Chemistry; IUPAC), an oxidation product of chlorophyll *a*. The structural difference between chlorophyll *d* and chlorophyll *a* is based on the presence of a formyl group instead of a vinyl group at the C-3 position of the macrocycle porphyrin ring in chlorophyll *d* (Figure 1). Holt and Morley (1959) were unsuccessful in isolating chlorophyll *d* from extracts of algae from Nova Scotia. However, by oxidizing chlorophyll *a* (the 3-vinyl group) with potassium permanganate in an aqueous acetone solution, a pigment with visible absorption spectra that appeared to be identical to that of chlorophyll *d* was obtained.

To confirm Holt and Morley's (1959) results, Holt (1961) compared 2-desvinyl-2-formyl chlorophyll *a* to chlorophyll *d* isolated from *Mastocarpus papillatus*. Holt (1961) concluded, with respect to visible absorption spectra, reduction products, and chromatographic behavior, that the oxidation product possessed identical properties to

chlorophyll *d*. However, Holt (1961) indicated that until sufficient amounts of chlorophyll *d* were isolated, further distinction could not be established, and concluded that chlorophyll *d* was most likely not an artifact of extraction because the routine boiling process prior to methanol extraction could not cause the oxidation of the 2-vinyl group of chlorophyll *a*. Thus the issue regarding chlorophyll *d* being an artifact of extraction remained unresolved.

The distribution of chlorophyll *d* in red algae is reported to be rare and irregular (Strain 1958, Sagromsky 1958, Sagromsky 1960, Holt 1961, Allen 1966, Evstigneev and Cherkashina 1970 Meeks 1974). For example, 15 years after his initial study, Strain (1958) extensively surveyed red algae collected at Half Moon Bay, Pacific Grove, and Pebble Beach, California, and Oahu, Hawaii. From a total of 76 species of red algae analyzed, a large concentration of chlorophyll *d* was detected in only one species (*Rhodocorton rothii*), less substantial amounts in 6 species, and trace amounts in 27 species. The survey showed chlorophyll *d* to occur sporadically, and it appeared to be totally lacking in the "primitive" Rhodophyta (such as the Bangiales). In addition, no chlorophyll *d* was detected in some species, such as *Erythrophyllum delesserioides*, which were reported to possess high concentrations by Manning and Strain (1943). Strain (1958) suggested the variation in quantities of chlorophyll *d* might be caused by environmental conditions, such as light intensity.

The interest in chlorophyll *d* appeared to have faded in the early 1970's (Table 1), perhaps because of chlorophyll *d*'s rare distribution (Rowan 1989) and the difficulty in isolating this pigment in large quantities (Evstigneev and Cherkashina 1970). It was not

until recently, more than 20 years since the original studies, that the topic of chlorophyll *d* has again become of interest (Miyashita et al. 1996, Miyashita et al. 1997, Marquardt et al 1997, Schiller et al. 1997, Hu et al. 1998).

Miyashita et al. (1996), reported chlorophyll *d* from a newly isolated, oxygenic, photosynthetic prokaryote, *Acaryochloris marina*. The organism was isolated from a suspension of algae “squeezed out” from a colonial ascidian from the coast of the Palau Islands. Chlorophyll *d* was detected *in vivo*, and the ratio of chlorophyll *a* to chlorophyll *d* in the cell varied from 0.027 to 0.092. Chlorophyll *d* was reported to be the major chlorophyll in *A. marina*. Miyashita et al. (1996) suggested chlorophyll *d* might be involved in a unique light harvesting system because of the low ratio of chlorophyll *a* to chlorophyll *d* (Miyashita et al. 1996). The Miyashita et al. (1996) study generated new interest in chlorophyll *d*, especially its role in photosynthesis (Miyashita et al. 1997, Marquardt et al 1997, Schiller et al. 1997, Hu et al. 1998).

The purpose of this study was to revisit the unsettled questions regarding the distribution of chlorophyll *d* in red algae. The specific objectives of this study were to apply high resolution high pressure liquid chromatography (HPLC) and spectroscopic assays to assess and verify the presence of chlorophyll *d* in red algae, to determine if chlorophyll *d* is an artifact of extraction, and to assess the contribution of chlorophyll *d* to *in vivo* absorbance characteristics. This study also surveyed chlorophyll *d* in a wide range of red algae from central California.

METHODS AND MATERIALS

Field Sampling and Pigment Extraction

California Samples

Red algae were collected intertidally and subtidally in October 1995, June and September 1996, and March and August 1999 from Pebble Beach, Pigeon Point, Pacific Grove, Carmel, and Point Lobos, California (Figure 2 and Appendix). Brown and green algae were collected in March 1999 from Pacific Grove. In June 2000, two species of red algae and two species of green algae from 36 to 51 meters were collected from Ventura Rocks and East Pinnacle, California using the ROV Ventana. Algae were collected, placed in plastic bags, and transported from the field to the laboratory in an ice chest with seawater. In the laboratory, algae were placed in continuously running seawater tables, sorted and identified.

Once identified, pieces of thalli from each alga were dried with a paper towel, cut into smaller pieces, weighed, placed in individual cryo vials containing 90% acetone, and stored at -20°C for pigment extraction (at least 24 hours). Acetone (90%), rather than methanol (100%), was used for pigment extraction because the algae, when soaked in 90% acetone were easier to homogenize, and 90% acetone was more efficient in extracting chlorophylls (74-85%). After extraction, samples were disrupted with a modified glass homogenizer until fragmented to fine debris (10-15 seconds). Careful attention was taken to ensure samples did not become hot during homogenization. Homogenates were centrifuged for about 5 minutes at 3000 rpm, and stored at -20°C until

analysis by high pressure liquid chromatography (HPLC), as discussed below. Algae collected in October were not weighed or ground.

Mexico and Antarctica Samples

Three species of red algae (*Amphiroa beauvoisii*, *Hypnea johnstonii*, and *Laurencia pacifica*) were collected in June 1999 from Bahia Concepcion, Baja California Sur, Mexico. Samples were prepared in the same manner as the California samples and stored at -20°C until pigment analysis.

Two species of red algae (*Iridea* sp. and *Phyllophora* sp.) were collected in October 1993 from Cape Evans, Antarctica, and stored at -20°C. Algae were defrosted, prepared in the same manner as the California samples, and stored at -20°C until pigment analysis.

HPLC and Spectroscopy

Extracted pigments in 90% acetone were separated by HPLC. Samples were injected with a Gilson 231 autosampler onto a 25 cm C₁₈ column (5 µm particles, Rainin Microsorb). The method consists of an elution gradient of 85:15 (v/v) methanol: 0.5 M ammonium acetate (aqueous) solution, 100% acetonitrile, and 100% acetone, similar to the method used by Welschmeyer 1994 (Table 2). Solvents were delivered at 1.5 mL per minute (Varian 5060 pump) with a linear gradient between each time step. Eluting pigments from algae collected in October were detected at 690 nm on an ISCO V⁴ absorbance detector with a Kratos FS950 in-line fluorescence. Broad band fluorescence excitation and emission filters were used (Corning CS 2-64). All other samples were detected at 440 nm on the ISCO V⁴ absorbance detector and with a 470 nm excitation

interference filter and a broad band fluorescence emission filter (Corning CS 2-64) on the Kratos FS950 fluorometer. A 470 nm excitation interference filter was used to produce a "cleaner" fluorescence chromatogram where a more pronounced chlorophyll *d* peak could be observed.

Because the October samples were detected at 690 nm on the absorbance detector and all other samples at 440 nm, the instrument was standardized at both wavelengths with purified chlorophyll *a* and chlorophyll *d* so that quantitative comparisons could be made.

To identify the HPLC retention time of chlorophyll *d*, an extracted sample of *Prionitis lanceolata* was injected on the HPLC system and every peak was collected individually using a Gilson 201 fraction collector. Times for each peak were recorded. Fluorescence emission and excitation spectra of each fraction in HPLC eluent were determined using a Spex Fluorolog scanning spectrofluorometer. Fractions were scanned with a band pass of 9.425 nm (2.5 mm slit width). Fractions were identified according to their spectral characteristics and compared with published data (Manning and Strain 1943, Smith and Benitez 1955, Evstigneev and Cherkashina 1970, Lu et al. 1983, Rowan 1989). Based on this analysis, chlorophyll *d* exhibited a retention time of 17.4 minutes. Chlorophyll *d* elutes near chlorophyll *a* (which elutes at 18.5 minutes) in an area where a few peaks due to degradation products of chlorophyll *a* are present. For this reason, to ensure that the chlorophyll *d* peak was collected, five fractions starting at 16.7 minutes (each fraction lasting 0.3 minutes) were collected for all subsequent samples and analyzed on the spectrofluorometer to determine if chlorophyll *d* was present in that sample.

Quantification of Chlorophyll *d* and Chlorophyll *a*

Extracts of red algae were combined and concentrated using disposable C₁₈ solid-phase extraction (SPE) columns (Baker® SPE, 100 mg) according to Welschmeyer (1994). Extraction columns (0.8 mL nominal volume) were tempered with one volume of 100% methanol, followed by one volume of 100% acetone, and then two final volumes of Nanopure® water. The crude extract was diluted with an equivalent volume of water to reduce solvent strength (1:1, 90% acetone extract: water). The mixture was loaded on the disposable column and drawn through the solid phase under weak vacuum (~100 mm Hg). Concentrated pigments then were eluted with about 0.5 mL of 100% acetone.

The concentrated extract was analyzed by HPLC. Chlorophyll *a* and chlorophyll *d* peaks were fraction collected and the HPLC eluents were transferred into nitrogen purged 90% acetone using disposable C₁₈ SPE columns as above. Concentrations of chlorophyll *a* and chlorophyll *d* were calculated from absorbance spectra collected using a Hewlett Packard® 8452A diode array spectrophotometer with extinction coefficients of 87.67 g⁻¹ cm⁻¹ at 664 nm and 110.4 g⁻¹ cm⁻¹ at 696 nm, respectively (Smith and Benitez 1955, Jeffrey and Humphrey 1975). The extinction coefficient of chlorophyll *d* in diethyl ether was used because the extinction coefficient of chlorophyll *d* in 90% acetone has not been calculated. Response factors (peak area/μg pigment) for the HPLC for both chlorophylls were determined.

Degradation Experiment

A time-series storage experiment was conducted to determine if chlorophyll *d* was a product of oxidative degradation during routine storage. Extracted pigments from *Gelidium purpurascens/coulteri* (alga with the most chlorophyll *d* present in the October samples) were stored at -20°C and analyzed by HPLC once per week over a 4 week period. Extracts were maintained at -20°C between sampling times.

In Vivo Detection of Chlorophyll d

Whole cell *in vivo* absorption spectra of *Gelidium purpurascens*, *Mastocarpus papillatus*, and *Endocladia muricata* were determined using a Shimadzu® UV 3000 spectrophotometer with an integrating sphere. Because chlorophyll *d* is usually present in small quantities, fourth derivative spectra also were obtained with the algorithm program provided by the manufacturer. Fourth derivatives (computing the slope of the original curve four times) provide enhanced wavelength information from spectral data not apparent in the original spectra (Smith and Alberte 1994).

After *in vivo* spectra were determined, pieces of the same algae were weighed, placed in vials with 3 mL 90% acetone for pigment extraction, and ground with a modified glass homogenizer (as previously described). Absorption spectra of extracted pigments were determined using a Hewlett Packard® 8452A diode array spectrophotometer.

Manning and Strain (1943) determined the presence of chlorophyll *d* in red algae based on the ratio of optical densities at 665 and 700 nm (E_{665}/E_{700}). In the present

study, the E₆₆₅/E₇₀₀ ratio for *G. purpurascens* was calculated to compare with Manning and Strain's ratio value.

RESULTS

Most of the sampling occurred within three periods: October 1995, June 1996, and March 1999 (Appendix). Fifty-seven species of red algae, four species of green algae, and five species of brown algae from central California were analyzed. Chlorophyll *d* was not detected in any of the green or brown macroalgae. However, chlorophyll *d* was detected in crude extracts from 47 of the 57 species of red algae sampled (Table 3). The percentage of species containing chlorophyll *d* differed between the three sampling periods (Figure 3). In October, 36 of the 36 species analyzed had chlorophyll *d*; in June, 12 of the 29; and in March, four of the 22. Chlorophyll *d* was detected in one (*Amphiroa beauvoisii*) of the three species collected in Mexico, and in one (*Iridea* sp.) of the two species collected in Antarctica. Chlorophyll *d* also was detected in one of the deep water red algae (*Maripelta rotata*). Neither of the deep water green algae contained chlorophyll *d*.

Chlorophyll *d* concentrations ranged from 0.0030 to 27 μg of chlorophyll *d*/g wet-weight. Chlorophyll *a* concentrations ranged from 0.97 to 458 μg of chlorophyll *a*/g wet-weight. Weight ratios of chlorophyll *a* to chlorophyll *d* ranged from 6 to 19444 (g/g). Sixty nine percent of the algae analyzed had chlorophyll *a* to *d* ratios ranging from 10 to 400, 23% had ratios higher than 400, and only 8% had ratios less than 10 (Figure 4).

Chlorophyll *d* from *Gelidium purpurascens* in 90% acetone had an absorption maximum in the red region at 690 nm, and Soret maxima at 396 and 450 nm (Figure 5). The fluorescence emission spectrum indicated a band at 699 nm, and the fluorescence excitation spectrum had maxima at 447 and 391 nm (Figure 6). The absorption and

fluorescence maxima agree with those of Manning and Strain (1943), Smith and Benitez (1955), and Rowan (1989). The maximum E_{665}/E_{700} ratio for chlorophyll *a* was estimated to be 2.6, and for chlorophyll *d*, 0.56.

Degradation Experiment

The *Gelidium* sp. extract sample, which was analyzed once a week for 4 weeks, showed no apparent increase in the concentration of chlorophyll *d* relative to chlorophyll *a*. Chlorophyll *a* concentrations ranged from 41 to 54 μg of chlorophyll *a*/g wet-weight, and chlorophyll *d* concentrations ranged from 21 to 26 μg of chlorophyll *d*/g wet-weight. Ratios of chlorophyll *a* to *d* stayed constant, ranging from 15 to 19 (g/g).

In Vivo Detection of Chlorophyll d

Two different samples of *Gelidium purpurascens* were analyzed. One sample had chlorophyll *d*, and the other did not. Chlorophyll *d* was detected *in vivo* in the absorption spectrum of the *G. purpurascens* sample containing chlorophyll *d* (Figure 7). A fourth derivative analysis resulted in bands at 686 nm due to chlorophyll *a* and at 718 nm due to chlorophyll *d* (Figure 7). Both samples of *G. purpurascens* in 90% acetone indicated absorption maxima in the red region at 664 nm and at 432 in the Soret due to chlorophyll *a* (Figure 7). The sample containing chlorophyll *d* had an additional maximum at 692 nm and a slight "hump" at 450 nm due to chlorophyll *d* (Figure 7). The sample with chlorophyll *d* had chlorophyll *a* and *d* concentrations of 393 μg and 122 μg , respectively.

Absorption spectra of *Endocladia muricata* and *Mastocarpus papillatus* were compared to those of *G. purpurascens*. Neither the *in vivo* nor the acetone extract

absorption spectra of *E. muricata* and *M. papillatus* indicated the presence of chlorophyll *d* (Figure 8). However, HPLC analysis (a more sensitive analysis) indicated the presence of chlorophyll *d* in small quantities (5.4 µg) in *E. muricata*. Chlorophyll *d* was not detected in *M. papillatus* in HPLC analysis. Chlorophyll *a* concentrations for *E. muricata* and *M. papillatus* were 347 and 220 µg, respectively.

DISCUSSION

Chlorophyll *d* did not appear to be an artifact of the extraction process or a degradation product from extended storage. Extended extraction times resulted in no net change in the chlorophyll *a* to *d* ratios, indicating chlorophyll *d* was not produced artificially. These results agree with those of Strain (1966), who analyzed red algae from Australia using several extraction methods to confirm chlorophyll *d* was not produced as an artifact of extraction. Algae were dried for various periods of time, and pigments were extracted with different solvents, such as acetone, methanol and pyridine. There was no increase in the proportion of chlorophyll *a* to *d* with each treatment, again affirming that chlorophyll *d* is not an artifact of the extraction process.

Uncertainty over the *in vivo* existence of chlorophyll *d* in red algae has been reported by Holt and Morley (1959), Sagromsky (1960), Evstigneev and Cherkashina (1970), Rowan (1989), and Gantt (1990). However, in this study, chlorophyll *d* was detected in such a high quantity in the *Gelidium purpurascens* pigment extracts that an *in vivo* chlorophyll *d* signal was easily detected in tissues of *G. purpurascens*. The *in vivo* *G. purpurascens* chlorophyll *d* absorption maximum (718 nm) agrees with the maximum reported by Miyashita et al (1996, 1997), from the recently isolated marine prokaryote (*Acaryochloris marina*), which contains chlorophyll *d* as its dominant chlorophyll. It was determined, however, that chlorophyll *d* must be present in very high quantities (chlorophyll *a* to *d* ratios of approximately 6) in order to be detected using a spectrophotometer, even when using an integrating sphere.

HPLC analysis was a more sensitive analysis than spectrophotometric analysis in detecting chlorophyll *d*. The maximum chlorophyll *a* to *d* ratio detectable by spectrophotometer was approximately 50 (Manning and Strain 1943), and approximately 19000 by HPLC (present study). HPLC was more than three orders of magnitude more sensitive than a spectrophotometer. The use of simple spectrophotometric assays, such as those used by Manning and Strain (1943), may explain the numerous failures to detect chlorophyll *d* in previous red algal surveys.

Previous studies suggest that chlorophyll *d* is only present in the more advanced red algae, such as the Florideophyceae (Strain 1958, Rowan 1989). I found chlorophyll *d* in a wide range of red algae, from the most primitive, such as the Bangiales, to the more complex, such as the Ceramiales (Table 3). Forty-seven species of red algae from California contained chlorophyll *d*, and these included *Porphyra perforata* and *Smithora naiadum*, both members of the class Bangiophyceae (Table 3). Furthermore, some studies have had difficulty isolating chlorophyll *d* from species collected in places other than off the Pacific coasts (Bjornland 1984, Calabrese and Felicini 1973, Holt and Morley 1959, Sagromsky 1960a), suggesting chlorophyll *d* might be unique to Pacific red algae. In this study, however, chlorophyll *d* was present in algae collected from Antarctica and Mexico. Chlorophyll *d* appears to be ubiquitous; it was present in intertidal algae, as well as, algae found as deep as 51 meters.

The distribution of chlorophyll *d* may have a seasonal signal; chlorophyll *d* was detected in all of the red algae analyzed (36 species) in October, 41% in June, and only 18% in March. Perennial red algae in central California appear to have a seasonal growth

pattern, with maximum cover in the summer and early fall, and minimum cover in the winter and early spring (Foster and Schiel 1985, Foster et al. 1988, Foster personal communication). These abundance changes might be related to a cycle of winter storm damage followed by new growth (Foster and Schiel 1985, Foster et al. 1988, Foster personal communication). In this study, the direct cause of difference in percentages of algae containing chlorophyll *d* might be due to several interacting factors, including variation in light, nutrients and temperatures. Sagromsky (1958, 1960) suggested that the variability of chlorophyll *d* within species depends on light intensity.

Sagromsky (1960) investigated 4 red algae collected at the Gulf of Naples in March and June. He reported 3 of the 4 algae contained chlorophyll *d* (3%, 3-6% and 28% of the total chlorophyll). The alga with the greatest content of chlorophyll *d*, *Nitophyllum punctatum*, was collected in June, during a period of high light in the Gulf of Naples. Sagromsky (1960) concluded that the presence of chlorophyll *d* could be attributed to light intensity, as an artifact of the extraction process, or a degradation product of chlorophyll *a*. Sagromsky (1960) suggested that exposure to high light might convert part of chlorophyll *a* to chlorophyll *d* through photo oxidation. In this study, *Gelidium purpurascens*, the alga with the highest chlorophyll *d* content, was collected in October, when organisms generally are exposed to longer daylight than in the late winter and spring months.

Manning and Strain (1943) detected chlorophyll *d* in red algae based on the ratio of optical densities at 665 and 700 nm (E_{665}/E_{700}). The E_{665}/E_{700} ratios for pure chlorophyll *a* and pure chlorophyll *d* were 90 and 0.25, respectively. The E_{665}/E_{700} ratio

between these upper and lower limits indicated a mixture of both chlorophylls. Manning and Strain (1943) reasoned that chlorophyll *d* could only be reliably detected in samples with E_{665}/E_{700} ratios <50 ; the minimum observed E_{665}/E_{700} ratio (corresponding to the highest chlorophyll *a* to chlorophyll *d* ratio) was 15. In this study, the estimated E_{665}/E_{700} ratio for chlorophyll *a* was 2.6, and 0.56 for chlorophyll *d*. The minimum observed E_{665}/E_{700} ratio was approximately 2, (in *Gelidium purpurascens*; Figure 7). The chlorophyll *d* peak in crude extracts from *G. purpurascens* was distinct compared to the chlorophyll *d* shoulder in *Erythrophyllum delesserioides* found by Manning and Strain (1943) (Figures 7 and 9).

The maximum minimum chlorophyll *a* to *d* ratio detected in this study was 6 (g/g). Sixty nine percent of the red algae had chlorophyll *a* to *d* ratios ranging from 10 to 400 (g/g), 23% had ratios higher than 400 (g/g), and approximately 8% of the algae contained ratios less than 6 (g/g) (Figure 4). The range of chlorophyll *a* to *d* ratio in this study was higher than ratios usually reported for accessory photosynthetic pigments in macroalgae. The chlorophyll *a* to *b* ratio is approximately 2:1 (Rowan 1989), and the chlorophyll *a* to *c* ratio is approximately 5:1 (Lee 1980). The high chlorophyll *a* to *d* ratios in the present study suggest chlorophyll *d* might be a specialized pigment in photosynthesis, similar to pheophytin *a*. Pheophytin *a* is only about 2% of the total chlorophyll *a* present and it is an essential component of Photosystem II in plants (Rowan 1989).

The role of chlorophyll *d* in photosynthesis is unclear. Given the inconsistency in isolating chlorophyll *d* in large quantities the role has been difficult to determine. Recent

studies on the action spectra of *Acaryochloris marina*, however, show chlorophyll *d* is involved in photosynthesis. The emission peak at 699 nm and the absorption peak longer than that of chlorophyll *a* (692 nm compared to 664 nm) suggest chlorophyll *d* may serve as a light-harvesting pigment in Photosystem I (Miyashita et al 1997, Marquardt et al 1997, Schiller et al. 1997, and Hu et al. 1998).

Acaryochloris marina is an unusual prokaryote that contains chlorophyll *d* as its major pigment, and chlorophyll *a* and phycobilins as minor pigments (Miyashita et al 1996, 1997). *Acaryochloris marina* was reported to have a Photosystem I reaction center driven by chlorophyll *d* (P740) that absorbs far-red light (Hu et al. 1998). Hu et al (1998) suggested the oxygenic photosynthesis based on chlorophyll *d* might be an acclimation to the far-red light that is not used by other organisms during photosynthesis. Hu et al (1998) also suggested it might be an evolutionary intermediate between the anoxygenic bacterial photosynthesis and the oxygenic plant/cyanobacterial photosynthesis.

Studies on *A. marina* (Miyashita et al 1997, Marquardt et al 1997, Schiller et al. 1997, and Hu et al. 1998) suggest there might be some connection between *A. marina* and red algae containing chlorophyll *d*. It is possible that *A. marina* is the predecessor to red algae that contain chlorophyll *d*, similar to the theory that cyanobacteria are the progenitor to red algae (Bonen and Doolittle 1975), or prochlorophytes are the progenitor to green algae (Lewin 1976).

In conclusion, chlorophyll *d* was found in many species of red algae, further corroborating its existence. Contrary to previous studies, chlorophyll *d* was found in the

primitive red algae, and it was not produced artificially. Chlorophyll *d* appears to be a seasonal pigment, which would explain why isolating it from red algae has been challenging. Further, chlorophyll *d* also appears to be a specialized pigment because it is found in very low amounts.

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Table 1. Summary of chlorophyll *d* studies.

Studies pertaining to chlorophyll <i>d</i>	
Study	Algae with Chlorophyll <i>d</i> Place of Collection
Manning and Strain 1943	10 out of 19 Moss Beach
Strain 1958	34 out of 76 Central California & Hawaii
Holt and Morley 1959	0 out of 3 Ireland and Nova Scotia
Sagromsky 1960a	3 out of 4 Gulf of Naples
Sagromsky 1960b	0 out of 16 Gulf of Naples
Holt 1961	2 out of 2 Moss Beach
Evstigneev and Cherkashina 1970	5 out of 10 Black Sea

Miscellaneous studies mentioning chlorophyll <i>d</i>	
Study	Algae with Chlorophyll <i>d</i> Place of Collection
Duysens 1951	1 out of 2 not reported
Smith & Benitez 1955	1 out of 1 Moss Beach
Strain 1966	24 species analyzed; most contained traces Australia
Calabrese & Felicini 1973	0 out of 1 Adriatic Sea
Bjornland & Aguilar-Martinez 1976	0 out of 8 Norway
Liu, et al. 1980	0 out of 1 Taiwan
Bjornland 1984	0 out of 1 Norway
Hegazi, et al. 1998	1 out of 1 Mar Menor Lagoon, SE Spain

Table 2. Timing of the solvent gradient.

Time (min)	Solvent A	Solvent B	Solvent C
	%		
0	100	0	0
8	0	100	0
13	0	70	30
17	0	20	80
25	0	20	80
26	100	0	0
33	100	0	0

Solvent A=85:15 MeOH:0.5 M ammonium acetate (aqueous)

Solvent B=100% acetonitrile

Solvent C=100% acetone

Table 3. Comparison of algae analyzed in 1958 by Strain to those analyzed in this study.

+ = chlorophyll d present

0 = chlorophyll d absent

Class	Order	Algae	Strain 1958	This study
Bangiophyceae	Bangiales	Bangia vermicularis		0
		Porphyra naiadum	0	
		Porphyra perforata	0	+
		Porphyra sp.		+
		Porphyridium cruentum	0	
		Porphyridium sp.		0
		Smithora naiadum		+
Florideophyceae	Nemaliales	Cumagloia andersonii	+	+
		Gelidium aborescens		0
		Gelidium coulteri	+	
		Gelidium purpurecence/coulteri		+
		Gelidium purpurescens		+
		Gelidium robustum		+
		Nemalion helminthoides		+
		Pseudogloiophloea confusa	0	
		Rhodochorton rothii	+	
Florideophyceae	Corallinales	Bossiella californica		0
		Bossiella corymbifera	+	
		Bossiella orbigniana	+	
		Bossiella plumosa	+	
		Calliarthron cheilosporioides	0	
		Calliarthron sp.		+
		Calliarthron tuberculosum	0	+
		Corallina officinalis var. chilensis	+	
		Corallina vancouveriensis		+
		Haliptylon gracile	+	
		Pseudolithophyllum neofarlowii	0	
Florideophyceae	Gigartinales	Callophyllis marginifructa	0	
		Callophyllis pinnata		+
		Chondracanthus canaliculatus	0	+
		Chondracanthus corymbiferus	0	+
		Chondracanthus spinosus	0	+
		Cryptosiphonia woodii	+	+
		Endocladia muricata	0	+
		Erythrophyllum delesserioides	0	
		Farlowia compressa	0	
		Farlowia mollis	0	
		Gracilaria lemaneiformis	+	+
		Grateloupia californica	0	
		Grateloupia setchellii	0	

Table 3 (cont.)

Class	Order	Algae	Strain 1958	This study
		<i>Gymnogongrus linearis</i>		+
		<i>Mastocarpus jardinii</i>	+	+
		<i>Mastocarpus papillatus</i>		+
		<i>Mazzaella affinis</i>		+
		<i>Mazzaella californica</i>	0	
		<i>Mazzaella flaccida</i>	0	+
		<i>Mazzaella heterocarpa</i>	+	
		<i>Mazzaella leptorhynchos</i>		+
		<i>Neoagardhiella baileyi</i>	0	
		<i>Opuntiella californica</i>		+
		<i>Plocamium cartilagineum</i>	+	+
		<i>Plocamium violaceum</i>	0	
		<i>Prionitis australis</i>	+	
		<i>Prionitis lanceolata</i>	+	+
		<i>Prionitis lyallii</i>	+	
		<i>Prionitis</i> sp.		+
		<i>Rhodoglossum californicum</i>	0	
		<i>Sarcodiotheca furcata</i>		
		<i>Sarcodiotheca gaudichaudi</i>		+
		<i>Weeksia reticulata</i>		+
Florideophyceae	Rhodymeniales	<i>Botryocladia pseudodichotoma</i>		+
		<i>Fauchia laciniata</i>	0	
		<i>Fryeella gardneri</i>		+
		<i>Gastroclonium subarticulatum</i>	0	+
		<i>Halosaccion glandiforme</i>	+	
		<i>Maripelta retata</i>		+
		<i>Rhodymenia pacifica</i>		+
Florideophyceae	Ceramiales	<i>Callithamnion acutum</i>	+	
		<i>Callithamnion pikeanum</i>		
		<i>Centroceras clavulatum</i>	+	
		<i>Ceramium eatonianum</i>	0	
		<i>Cryptopleura farlowianum</i>	0	+
		<i>Cryptopleura lobulifera</i>	+	0
		<i>Cryptopleura</i> sp 1 (lobulifera?)		0
		<i>Cryptopleura</i> sp 2 (lobulifera?)		0
		<i>Cryptopleura violacea</i>	+	
		<i>Erythrocytis saccata</i>	+	+
		<i>Griffithsia pacifica</i>	+	
		<i>Hymenena flabelligera</i>	+	
		<i>Hymenena kylinii</i>	+	
		<i>Hymenena multiloba</i>	+	+
		<i>Laurencia pacifica</i>	+	+

Table 3 (cont.)

Class	Order	Algae	Strain 1958	This study
		Laurencia subopposita		+
		Microcladia borealis	0	+
		Microcladia coulteri	0	+
		Neoptilota densa	0	
		Neorhodomela larix	+	+
		Nienburgia andersoniana	+	
		Odonthalia floccosa	0	
		Osmundea spectabilis	0	+
		Polyneura latissima	0	
		Polysiphonia paniculata	0	
		Polysiphonia hendryi	0	
		Polysiphonia scopulorum var. villun	0	
		Polysiphonia sp.		+
		Pterosiphonia baileyi	0	
		Pterosiphonia bipinnata	+	
		Pterosiphonia dendroidea	+	
		Rhodoptilum plumosum		+
		Tiffaniella snyderae	+

Figure 1. Chemical structures of chlorophyll *a* and *d* (from Hu et al. 1998)

Figure 2. Map of sampling locations.

Figure 3. Percentage of species of red algae with chlorophyll *d*.

Figure 4. Frequency of ratio of chlorophyll *a* to *d* (g/g).

Figure 5. Absorption spectrum of chlorophyll *d* in 90% Acetone.

Figure 6. a) Fluorescence emission spectrum of chlorophyll *d* in 90% acetone; b) Fluorescence excitation spectrum of chlorophyll *d* in 90% acetone.

Figure 7. a) *In vivo* absorption spectra of *G. purpurascens* (spectra normalized to 676 nm); b) Fourth derivative of *G. purpurascens*; c) Absorption spectra of *G. purpurascens* in 90% acetone (spectra normalized to 664 nm)

Figure 8. a) *In vivo* absorption spectra of *E. muricata* and *M. papillatus* (spectra normalized to 676 nm); b) Fourth derivative of *in vivo* *E. muricata* and *M. papillatus*; c) Absorption spectra of *E. muricata* and *M. papillatus* in 90% acetone (spectra normalized to 664 nm).

Figure 9. Absorption curves for pigments in methanol extracts of *Erythrophyllum delesserioides* (redrawn from Manning and Strain 1943).

Appendix 1. List of algae collected in central California

Species	Date Collected	Place of Collection
Reds		
<i>Botryocladia pseudodichotoma</i>	10/19/95	Stillwater (35ft)
<i>Calliarthron tuberculosum</i>	10/19/95	Stillwater (35ft)
<i>Cryptopleura farlowianum</i>	10/19/95	Stillwater (35ft)
<i>Fryeella gardneri</i>	10/19/95	Stillwater (35ft)
<i>Gelidium robustum</i>	10/19/95	Stillwater (35ft)
<i>Chondracanthus corymbiferus</i>	10/19/95	Stillwater (35ft)
<i>Chondracanthus spinosus</i>	10/19/95	Stillwater (35ft)
<i>Gymnogongrus linearis</i>	10/19/95	Stillwater (35ft)
<i>Osmundea spectabilis</i>	10/19/95	Stillwater (35ft)
<i>Microcladia coulteri</i>	10/19/95	Stillwater (35ft)
<i>Opuntiella californica</i>	10/19/95	Stillwater (35ft)
<i>Plocamium cartilagineum</i>	10/19/95	Stillwater (35ft)
<i>Prionitis lanceolata</i>	10/19/95	Stillwater (35ft)
<i>Rhodomenia pacifica</i>	10/19/95	Stillwater (35ft)
<i>Smithora naiadum</i>	10/19/95	Stillwater (35ft)
<i>Corallina vancouveriensis</i>	10/20/95	Stillwater (intertidal)
<i>Cumagloia andersonii</i>	10/20/95	Stillwater (intertidal)
<i>Endocladia muricata</i>	10/20/95	Stillwater (intertidal)
<i>Gastroclonium subarticulatum</i>	10/20/95	Stillwater (intertidal)
<i>Chondracanthus canaliculatus</i>	10/20/95	Stillwater (intertidal)
<i>Osmundea spectabilis</i>	10/20/95	Stillwater (intertidal)
<i>Laurencia subopposita</i>	10/20/95	Stillwater (intertidal)
<i>Mastocarpus papillatus</i>	10/20/95	Stillwater (intertidal)
<i>Nemalion helminthoides</i>	10/20/95	Stillwater (intertidal)
<i>Mazzaella affinis</i>	10/20/95	Stillwater (intertidal)
<i>Smithora naiadum</i>	10/20/95	Stillwater (intertidal)
<i>Callophyllis pinnata</i>	10/23/95	Pigeon Point (intertidal)
<i>Cryptosiphonia woodii</i>	10/23/95	Pigeon Point (intertidal)
<i>Gelidium purpureascens</i>	10/23/95	Pigeon Point (intertidal)
<i>Gracilaria lemaneiformis</i>	10/23/95	Pigeon Point (intertidal)
<i>Hymenena multiloba</i>	10/23/95	Pigeon Point (intertidal)
<i>Mazzaella flaccida</i>	10/23/95	Pigeon Point (intertidal)
<i>Microcladia borealis</i>	10/23/95	Pigeon Point (intertidal)
<i>Polysiphonia</i> sp.	10/23/95	Pigeon Point (intertidal)
<i>Porphyra</i> sp.	10/23/95	Pigeon Point (intertidal)
<i>Neorhodomela larix</i>	10/23/95	Pigeon Point (intertidal)
<i>Rhodoptilum plumosum</i>	10/23/95	Pigeon Point (intertidal)

Appendix 1 (cont.)

Species	Date Collected	Place of Collection
<i>Sarcodiotheca furcata</i>	10/23/95	Pigeon Point (intertidal)
<i>Sarcodiotheca gaudichaudii</i>	6/17/96	Pacific Grove (intertidal)
<i>Prionitis lanceolata</i>	6/17/96	Pacific Grove (intertidal)
<i>Mazzaella affinis</i>	6/17/96	Pacific Grove (intertidal)
<i>Chondracanthus spinosus</i>	6/17/96	Pacific Grove (intertidal)
<i>Chondracanthus corymbiferus</i>	6/17/96	Pacific Grove (intertidal)
<i>Chondracanthus canaliculatus</i>	6/17/96	Pacific Grove (intertidal)
<i>Gelidium purpureascens</i>	6/17/96	Pacific Grove (intertidal)
<i>Gastroclonium subarticulatum</i>	6/17/96	Pacific Grove (intertidal)
<i>Mazzaella leptorhynchos</i>	6/17/96	Pacific Grove (intertidal)
<i>Gelidium robustum</i>	6/17/96	Pacific Grove (intertidal)
<i>Smithora naiadum</i>	6/17/96	Pacific Grove (intertidal)
<i>Cryptopleura farlowianum</i>	6/17/96	Pacific Grove (intertidal)
<i>Calliarthron tuberculosum</i>	6/17/96	Pacific Grove (intertidal)
<i>Corallina vancouveriensis</i>	6/17/96	Pacific Grove (intertidal)
<i>Cumagloia andersonii</i>	6/19/96	Stillwater (intertidal)
<i>Porphyra perforata</i>	6/19/96	Stillwater (intertidal)
<i>Cryptopleura lobulifera</i>	6/19/96	Stillwater (intertidal)
<i>Polysiphonia sp.</i>	6/19/96	Stillwater (intertidal)
<i>Osmundea spectabilis</i>	6/19/96	Stillwater (intertidal)
<i>Endocladia muricata</i>	6/19/96	Stillwater (intertidal)
<i>Nemalion helminthoides</i>	6/19/96	Stillwater (intertidal)
<i>Gracilaria lemaneiformis</i>	6/19/96	Stillwater (intertidal)
<i>Laurencia pacifica</i>	6/19/96	Stillwater (intertidal)
<i>Erythrocytis saccata</i>	6/19/96	Stillwater (intertidal)
<i>Bossiella californica</i>	6/26/96	Stillwater (35-39ft)
<i>Laurencia subopposita</i>	6/26/96	Stillwater (35-39ft)
<i>Plocamium cartilagineum</i>	6/26/96	Stillwater (35-39ft)
<i>Fryeella gardneri</i>	6/26/96	Stillwater (35-39ft)
<i>Weeksia reticulata</i>	6/26/96	Stillwater (35-39ft)
<i>Bangia vermicularis</i>	9/8/96	Stillwater (intertidal)
<i>Mastocarpus papillatus</i>	9/8/96	Stillwater (intertidal)
<i>Gelidium purpureascens/coulteri</i>	3/16/99	Pacific Grove (intertidal)
<i>Gelidium aborescens</i>	3/16/99	Pacific Grove (intertidal)
<i>Corallina vancouveriensis</i>	3/16/99	Pacific Grove (intertidal)
<i>Endocladia muricata</i>	3/16/99	Pacific Grove (intertidal)
<i>Prionitis sp.</i>	3/16/99	Pacific Grove (intertidal)
<i>Gracilaria lemaneiformis</i>	3/16/99	Pacific Grove (intertidal)

Appendix 1 (cont.)

Species	Date Collected	Place of Collection
<i>Mastocarpus papillatus</i>	3/16/99	Pacific Grove (intertidal)
<i>Chondracanthus canaliculatus</i>	3/16/99	Pacific Grove (intertidal)
<i>Mastocarpus jardinii</i>	3/16/99	Pacific Grove (intertidal)
<i>Mazzaella leptorhynchus</i>	3/16/99	Pacific Grove (intertidal)
<i>Chondracanthus spinosus</i>	3/16/99	Pacific Grove (intertidal)
<i>Mazzaella flaccida</i>	3/16/99	Pacific Grove (intertidal)
<i>Porphyra perforata</i>	3/16/99	Pacific Grove (intertidal)
<i>Chondracanthus affinis</i>	3/16/99	Pacific Grove (intertidal)
<i>Gastroclonium subarticulatum</i>	3/16/99	Pacific Grove (intertidal)
<i>Microcladia borealis</i>	3/16/99	Pacific Grove (intertidal)
<i>Microcladia coulteri</i>	3/16/99	Pacific Grove (intertidal)
<i>Cryptopleura sp 1</i>	3/16/99	Pacific Grove (intertidal)
<i>Cryptopleura sp 2</i>	3/16/99	Pacific Grove (intertidal)
<i>Polysiphonia sp.</i>	3/16/99	Pacific Grove (intertidal)
<i>Osmundea spectabilis</i>	3/16/99	Pacific Grove (intertidal)
<i>Calliarthron sp.</i>	3/16/99	Pacific Grove (intertidal)
<i>Opuntiella californica</i>	8/28/99	Point Lobos (27m)
<i>Weeksia reticulata</i>	8/28/99	Point Lobos (27m)
<i>Maripelta retata</i>	6/2/00	Ventura Rocks (51m)
<i>Rhodymenia sp.</i>	6/1/00	East Pinnacle (47m)
Greens		
<i>Chetomorpha sp.</i>	3/16/99	Pacific Grove (intertidal)
<i>Cladophora columbiana</i>	3/16/99	Pacific Grove (intertidal)
<i>Codium sp.</i>	6/1/00	East Pinnacle (36m)
<i>Unknown green</i>	6/1/00	East Pinnacle (38m)
Browns		
<i>Cylindrocarpus rugosus</i>	3/16/99	Pacific Grove (intertidal)
<i>Colpomenia peregrina</i>	3/16/99	Pacific Grove (intertidal)
<i>Egregia menziesii</i>	3/16/99	Pacific Grove (intertidal)
<i>Pelvetia fastigiata</i>	3/16/99	Pacific Grove (intertidal)
<i>Hesperophycus harveyanus</i>	3/16/99	Pacific Grove (intertidal)