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NOTE

QUANTIFYING GROWTH AND CALCIUM CARBONATE DEPOSITION OF *CALLIARTHRON CHEILOSPORIOIDES* (CORALLINALES, RHODOPHYTA) IN THE FIELD USING A PERSISTENT VITAL STAIN[1]

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Growth and calcium carbonate deposition rates of the coralline alga *Calliarthron cheilosporioides* Manza were quantified by monitoring fronds in the intertidal zone that had been chemically labeled with the nontoxic fluorescent brightener Calcofluor white. This vital stain effectively labeled apical meristems of coralline thalli in the field: fronds exposed for only 5 min had detectable chemical marks at least 1.5 years later. By distinguishing portions of thalli that developed before and after exposure, this methodology permitted accurate measurement of growth and calcium carbonate deposition at each meristem. In *Calliarthron*, meristematic activity declined with increasing frond size. However, because growing fronds dichotomize, the total number of meristems and the deposition rate of new calcified tissue both increased with frond size. Growth rates reported here suggest that large fronds may not be as old as previously estimated. The Calcofluor white method may improve demographic studies of corallines by resolving growth and age of fronds in the field and may facilitate studies of climate change on calcium carbonate deposition in these ecologically important, calcifying algae.

Key index words: **calcification; calcium carbonate ($CaCO_3$); Calcofluor white; conceptacle; coralline algae; demography; growth rate; intertidal; macroalgae; meristem**

Abbreviation: **$CaCO_3$, calcium carbonate**

Coralline algae (Rhodophyta, Corallinaceae) are important components of marine communities worldwide (Littler 1972, Adey 1975, Foster 1975, Johansen 1981, Paine 1984, Steneck 1986). They are early colonizers of bare substrata (Matsuda 1989, Kendrick 1991, P. T. Martone, pers. obs.), dominant competitors for space (Quinn 1982, Paine 1984, Steneck 1986, Steneck and Paine 1986), and prominent members of most wave-swept intertidal flora (Johansen 1981). Corallines cement coral reefs together (Gardiner 1931, Adey 1975, Steneck and Adey 1976, Johansen 1981), induce settlement of invertebrate larvae (Gee 1965, Rumrill and Cameron 1983, Sebens 1983, Kitamura et al. 2007, Williams et al. 2008), and provide habitat for diverse meiofaunal communities (Dommasnes 1968, Akioka et al. 1999, Kelaher 2002, Steller et al. 2003). Despite the ubiquity of coralline algae and the central role they play in ecological processes, demographic studies of corallines in the field—especially those living in the rocky intertidal zone—are few.

Growth rates of coralline algae are generally regarded as being slow (Johansen 1981, Steneck 1986), perhaps hampered by the heavy deposition of $CaCO_3$ into calcifying cell walls. Marginal extension rates for encrusting corallines may exceed 2–3 mm · month^{-1} (Gardiner 1931, Adey and Vassar 1975, Steneck 1985) but are typically <1 mm · month^{-1} (Littler 1972, Steneck and Adey 1976, Johansen 1981, Matsuda 1989). Exceptionally slow accretion rates of 0.02–2.5 mm · year^{-1} suggest that some large encrusting and free-living rhodolith species may live 100 to 1,000 years (Adey and McKibbin 1970, Adey and MacIntyre 1973, Blake and Maggs 2003, Rivera et al. 2004, Frantz et al. 2005, Kamenos et al. 2008). Erect articulated corallines may grow much faster, up to 5 mm · month^{-1} (Haas et al. 1935, Smith 1972), but on average grow 1.5–2 mm · month^{-1} (Johansen and Austin 1970, Pearse 1972, Andrake and Johansen 1980, Blake and Maggs 2003). Large (>10 cm tall) articulated fronds produced by representatives of the genus *Calliarthron* are estimated to be 5–10 years old (Johansen and Austin 1970, Foster 1975).

Measuring macroalgal growth in the field can be difficult, requiring individual fronds to be monitored through time. One noninvasive method for documenting frond development is to analyze photographs taken repeatedly of experimental fronds. However, growth estimates based solely on comparisons of photographs taken in the field (Johansen and Austin 1970) are likely to be imprecise. To improve repeated measurements, past studies have marked or labeled fronds in the field. For example,

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zip-ties (Klinger and DeWreede 1988) and hole-punches (Koehl et al. 2008) have been used to physically label and monitor growth of large kelps. However, physically labeling small algal fronds is complicated and may damage delicate thalli, especially under breaking waves in the intertidal zone. As an alternative to physical labeling, several researchers chemically labeled coralline fronds using the vital stain Alizarin red (Andrake and Johansen 1980, Agegian 1981, 1985, Blake and Maggs 2003, Rivera et al. 2004, Steller et al. 2007, Halfar et al. 2008) but with mixed success; chemical marks sometimes faded from coralline surfaces in the field (Andrake and Johansen 1980, Blake and Maggs 2003), limiting their utility. Given the difficulties inherent in field experimentation, many studies on coralline growth and calcium carbonate deposition have, instead, been conducted in the laboratory (Goreau 1963, Adey 1970, Pearse 1972, Smith 1972) or on artificial substrata (Adey and Vassar 1975, Matsuda 1989) and, therefore, may not accurately reflect performance in the natural environment.

In this study, growth and calcium carbonate deposition rates were measured in the intertidal coralline *C. cheilosporioides* by monitoring chemically labeled fronds in the field through time. Coralline fronds were stained with Calcofluor white, a nontoxic fluorescent brightener that has been used to label algal cell walls to explore gametogenesis, rhizoid development, and cell-wall extension (Cole 1964, Nakazawa et al. 1969, Hsaio and Druehl 1973, Waaland and Waaland 1975). Fronds stained with Calcofluor white in the field retained chemical marks for at least 1.5 years, establishing this method as a useful tool for documenting growth under natural conditions.

Fronds of *C. cheilosporioides* were stained in the wave-exposed rocky intertidal zone at Hopkins Marine Station in Pacific Grove, California (36°36′ N, 121°53′ W). During low tide on 14 June 2006, fronds emerging from four thalli on vertical rock faces were dipped into a 0.02% solution of Calcofluor white (Sigma-Aldrich, St. Louis, MO, USA, Fluorescent brightener 28) held in a one-quart plastic bag and submersed for ~5 min. Due to the hazards of crashing waves and the flooding of solution bags, it was not possible to increase the duration of stain application on this date. Single fronds selected at random from labeled thalli were collected during subsequent low tides on 26 June 2006, 25 July 2006, 10 August 2006, 4 November 2006, and 8 January 2008 (i.e., 12, 41, 57, 143, and 573 d after stain application) and transported to the laboratory for analysis.

Stained fronds were placed under an ultraviolet lamp (Phillips Lighting, Amsterdam, the Netherlands, bulb# TLD 15W/08, 315–400 nm) and digitally photographed (Canon Inc., Tokyo, Japan, EOS Rebel XT) with a long exposure (~10 s). Fluorescent marks were not visible to the naked eye but were apparent in the photographs (Fig. 1). Fluorescent marks corresponded to the position of apical meristems at the time of stain application, suggesting that these regions of active growth are highly receptive to Calcofluor white.

Images were analyzed using the ImageJ software (v 1.36b, National Institutes of Health, Bethesda, MD, USA). Growth rates were quantified by measuring the length and planform area (i.e., half the wetted surface area) of newly deposited tissue distal to fluorescent chemical marks and dividing by the time elapsed since stain application. Five randomly selected branches were measured in each frond to yield an average growth rate. The planform area of fronds at the time of stain application was measured by deducting the total area of new growth from the planform area of each frond at collection. Loss of calcified segments over time was not quantified but was assumed to be negligible given that most meristems appeared intact. Growth rates were plotted as functions of original planform area of fronds.

To explore the rate of calcium carbonate deposition, $CaCO_3$ content was first measured in 10 fronds collected from the same field site. Fronds were dried, weighed, decalcified overnight in 1N HCl, and then redried and reweighed. The mass of $CaCO_3$ was calculated from the difference between dried mass and decalcified dried mass. With little variation, fronds were $84.7 \pm 0.2\%$ $CaCO_3$ (mean ± standard error of mean). The dried mass of fluorescently labeled fronds was measured, and the mass of $CaCO_3$ content was estimated proportionally. Because fronds grow primarily in a single plane and maintain a nearly constant thickness, $CaCO_3$ content was significantly correlated with frond planform area [$Y = 54.96\ X$, where Y is the mass of $CaCO_3$ (mg), and X is the frond area (cm^2); $r^2 = 0.94$, $P < 0.001$]. Given that $CaCO_3$ content varies little among new coralline segments (Pearse 1972), the equation above was used to convert rate of change in planform area to rate of $CaCO_3$ deposition by individual meristems and by entire fronds. To explore reproductive investment, the total number of conceptacles produced by each frond since stain application was counted and plotted against the original planform area of fronds. Regression statistics were computed using JMP software (Version 7.0.2, SAS Institute, Cary, NC, USA).

Calcofluor white quickly and effectively stained meristems in fronds of *C. cheilosporioides* in the field, demonstrating the utility of this vital stain for use in field studies. Plants stained for just 5 min maintained detectable fluorescent marks >1.5 years (573 d) after exposure (Fig. 1), suggesting that this short soak time may be sufficient to stain and monitor fronds indefinitely.

Growth and $CaCO_3$ deposition rates of meristems declined with increasing frond size (Fig. 2, A and B). Individual meristems in small fronds (<5 cm^2)

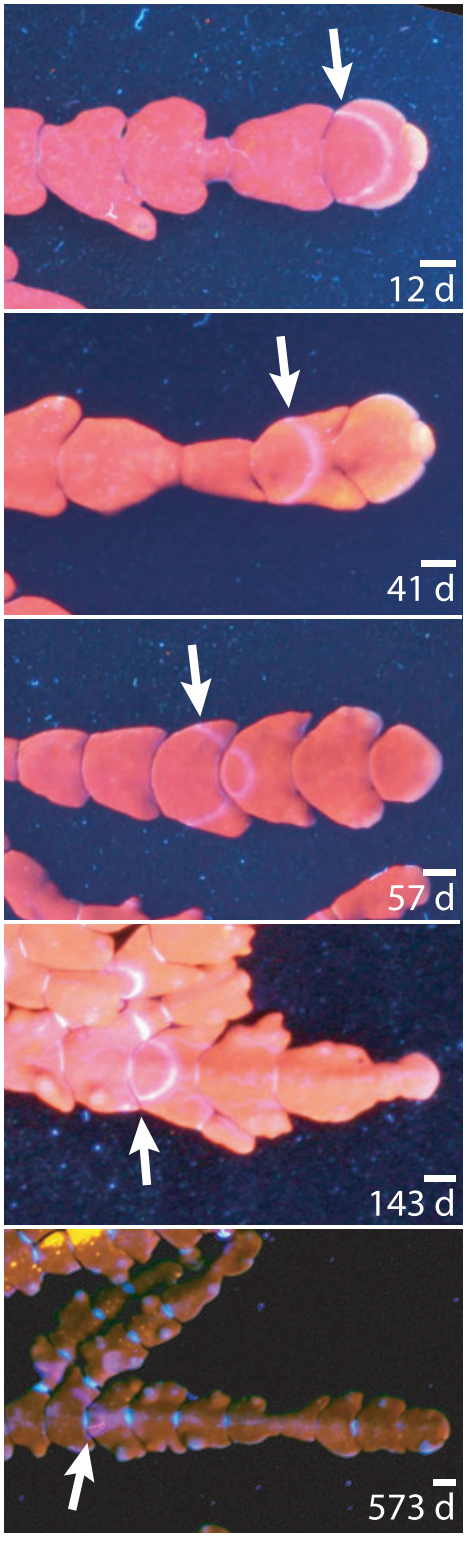

FIG. 1. Calcofluor white effectively labeled the apical meristem of growing fronds. The fluorescent mark (see arrows) was detectable for at least 1.5 years, providing a precise measure of growth since exposure. Depicted are five different fronds collected over the specified time period. Note that the autofluorescence of genicular tissue between calcified segments in the last image is distinct from the fluorescent chemical mark. Scale bars, 1 mm.

grew ~2.3–3.3 mm in length per month, while those in larger fronds (>15 cm^2) grew roughly half as fast: 0.8–1.6 mm · $month^{-1}$ (Fig. 2A). These growth rates are comparable with those reported for congeners growing subtidally (Johansen and Austin 1970), suggesting that physical stresses in the intertidal habitat may not significantly affect growth or that environmental stresses may be offset by increased opportunity (e.g., increased light and nutrient delivery). In terms of area, individual meristems in small fronds (<5 cm^2) grew ~0.1–0.15 $cm^2 \cdot month^{-1}$, depositing 5.6–8.6 mg $CaCO_3$, while those in larger fronds (>15 cm^2) grew 0.02–0.04 $cm^2 \cdot month^{-1}$, depositing 1.2–2.2 mg $CaCO_3$ (Fig. 2B).

Although meristematic activity slows as fronds increase in size, growing fronds dichotomize, increasing the total number of apical meristems and thereby increasing the total rate of new tissue production. Using the Calcofluor white method, one can precisely document the growth of all meristems and, therefore, calculate total change in area and $CaCO_3$ deposited over time. Despite limited sample size, the data presented here suggest that small fronds grew ~0.25–0.75 cm^2 new area per month, depositing 15–40 mg $CaCO_3$, while larger fronds grew 1.2–1.8 cm^2 new area per month, depositing 65–95 mg $CaCO_3$ (Fig. 2C). Although change in length is the standard measure for quantifying growth in coralline crusts and fronds (Johansen and Austin 1970, Adey and Vassar 1975, Steneck and Adey 1976, Andrake and Johansen 1980, Matsuda 1989), change in planform area is more ecologically informative. Increased area represents increased light interception for photosynthesis, increased risk of breakage due to drag imposed by breaking waves (Martone and Denny 2008), increased $CaCO_3$ deposition, and increased reproductive potential. For example, the rate of conceptacle production increased with frond size (Fig. 3). On average, small fronds (<5 cm^2) produced ~4–10 new conceptacles per month, while larger fronds (>15 cm^2) produced >40 conceptacles per month (Fig. 3).

Because growth rates varied with frond size (Fig. 2), future studies on growth and $CaCO_3$ deposition will need to sample more fronds across a wider size range (e.g., 10 replicate fronds per 5 cm^2 size-class bin up to 40 cm^2) to generate representative growth curves for *Calliarthron*; consequently, the reader should view the regressions presented here with caution. Nevertheless, based on the data presented here, if we conservatively assume a constant growth rate of 0.5 $cm^2 \cdot month^{-1}$ (see Fig. 2C), we estimate that a large *Calliarthron* frond (40 cm^2, Martone and Denny 2008) would be 6.7 years old. A more realistic estimate of 1.0 $cm^2 \cdot month^{-1}$ (see Fig. 2C) would make the same frond 3.3 years old. If the trend depicted in Figure 2C continues, maximum growth rate may be even faster, and the frond younger. Although good estimates of size-at-age remain to

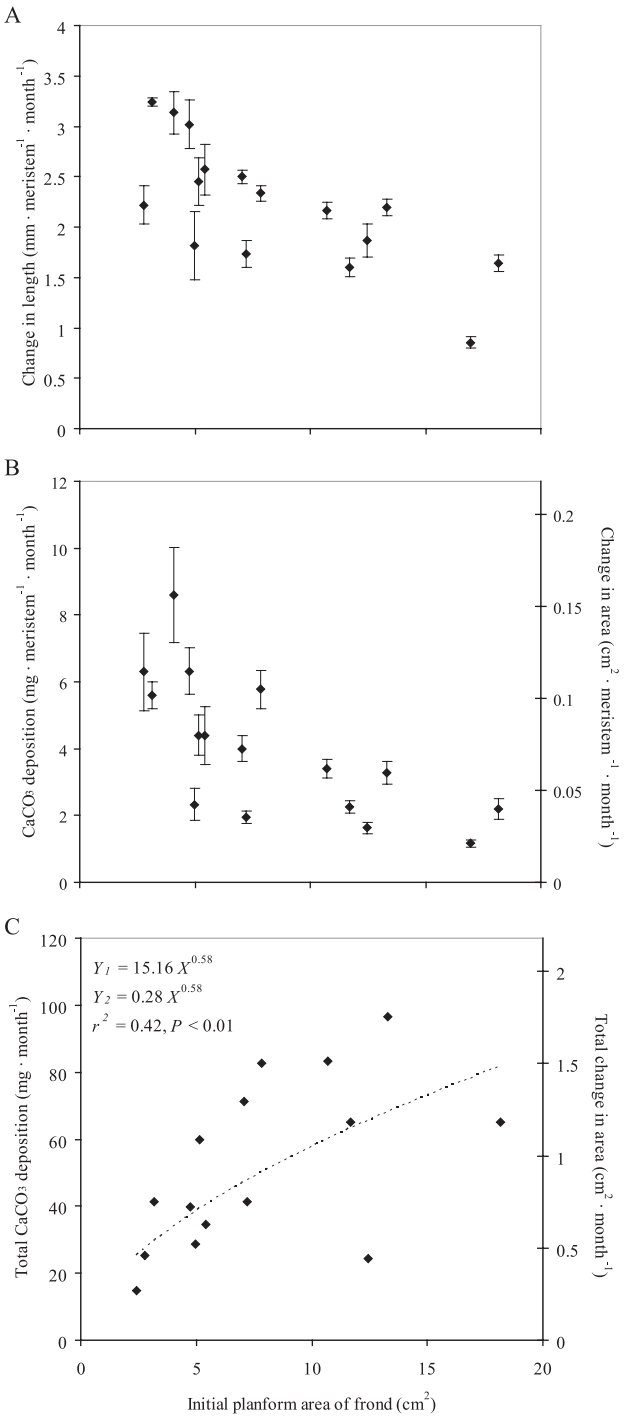

FIG. 2. Growth and $CaCO_3$ deposition rates as functions of frond planform area at the time of stain application. Per meristem, change in length (A), change in area, and $CaCO_3$ deposition (both shown in B) decline with increasing frond size. Data are mean ± standard error of mean ($N = 5$ meristems). Because larger fronds have more meristems, the total change in area and $CaCO_3$ deposited increases with frond size (C).

be determined for articulated corallines, results from this Calcofluor white study suggest that previous estimates of frond age (5–10 years: Johansen and Austin 1970, Foster 1975) may be overestimates.

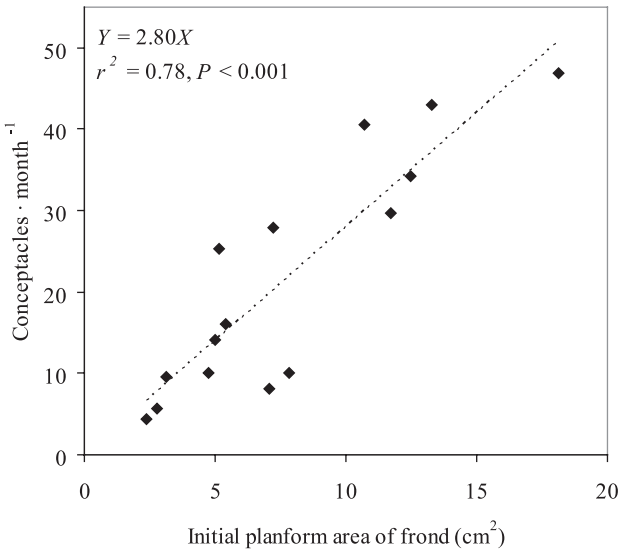

FIG. 3. Average number of conceptacles produced by *Calliarthron* fronds per month as a function of frond planform area at the time of stain application.

In addition to improving demographic studies of coralline algae in the field, Calcofluor white may be a useful tool for researchers studying the biological effects of climate change and ocean acidification (Hall-Spencer et al. 2008, Kuffner et al. 2008). For example, coralline algae are a distinct carbon sink; as they calcify, corallines fix carbon into a state that is not readily accessible by herbivores and, therefore, not immediately released back into the atmosphere (Smith 1972, Stearn et al. 1977). Given that a single large *Calliarthron* frond (40 cm^2) likely deposits >2 g of $CaCO_3$ in its lifetime, the total amount of carbon fixed by coralline populations along the shore may be significant. Understanding the rate of $CaCO_3$ deposited by *Calliarthron* and other corallines may, therefore, improve global carbon budgets. Furthermore, by clearly indicating regions of new growth, Calcofluor white will facilitate future experiments exploring effects of increased temperature, decreased pH, and other climatic shifts on coralline growth and calcification.

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