Measuring the growth of the articulated coralline red algae *Corallina officinalis* and *Arthrocardia corymbosa* (Corallinaceae, Rhodophyta) using a fluorescent brightener

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Abstract

Coralline algae are important components of the coastal marine ecosystem, making it crucial to understand influences on their production and their growth rate in general. A few attempts have been made to quantify coralline growth rate using various methods, however since they have been reported to be slow growers it is difficult to quantify their growth rate accurately. Calcofluor white is an optical brightener that can be used to stain cell walls of plants and has been used to stain articulated corallines in the field, which proved to be a useful and accurate method for measuring coralline growth rate. This study tested the use of Calcofluor white for measuring growth of articulated (geniculate) coralline algae found on the south-west coast of South Africa, and made a series of methodological tests on the use of the stain. The growth rate of *Corallina officinalis* and *Arthrocardia corymbosa* were compared by growing them in culture in aerated plastic bags at 15°C and 16 hr light:8hr dark. Only *C. officinalis* produced measurable growth in culture (0.08-0.09 mm day $^{-1}$). Further tests were carried out only on *C. officinalis.* Additionally, an attempt was made to stain *C. officinalis* in a rock pool, *in situ*. In culture, the exposure time to the dye was tested for effect on growth rate. Growth rates were similar after 8 days, but after 20 and 28 days thalli stained for 15 min grew significantly slower than those stained for 5 or 30 min. However, this effect seems to be caused by factors other than staining time (e.g. light or water movement in the culture bags). Growth in culture of *C. officinalis* was strongly effected by salinity: at 60% saltwater (±21 ppt) growth fell to 0.03 mm day $^{-1}$, and there was no growth in 30% saltwater (±10 ppt). The effects of 3 preservation methods on staining in *C. officinalis* were also tested. The stain was unaffected by drying the thalli or preserving them in 5% formalin in seawater. Preservation in 70% alcohol/20% glycerol/10% freshwater (a common method of preserving coralline for DNA analysis) reduced stain visibility, particularly when thalli had only been stained for 5 min. The study confirms that Calcofluor white is an effective fluorescent dye for staining coralline red algae. Furthermore, staining can be as short as 5 min and thalli can be preserved without affecting the stain, so that thalli can be stained *in situ*, preserved, and brought to the laboratory for measurement.

Key Words

South Africa, Calcofluor White, growth rate, macroalgae, culture, *in situ*

Introduction

Coralline red algae are present in almost all rocky coastal habitats and are important components of marine ecosystems worldwide (Johansen 1981). They flourish equally in cold polar as well as warm tropical seas and are commonly found in temperate waters. Not only do they cement coral reefs together by forming a hard crust over the soft limestone (Björk et al. 1995) but also provide habitat for meiofaunal communities (Johansen 1981, Brown and Taylor 1999, Kelaher 2002), induce invertebrate larvae settlement (Williams et al 2008), are competitive colonizers of bare substrata (Dye 1993, Goldberg and Foster 2002) and are one of the most important producers of calcium carbonate sand (Van den Hoek et al. 1995).

Corallines belong to the order *Corallinales* and family *Corallinaceae.* Two types of coralline thalli are recognised, the articulated and the nonarticulated or crustose (Bold and Wynne 1985). Articulated species consist of an erect portion with a segmented structure due to the alternation of calcified portions (intergenicula) with noncalcified portions (genicula). Their cell walls are encrusted with lime ($CaCo_3$), which occurs as a crystal or calcite (Van den Hoek et al. 1995). The geniculum gives them the ability of the otherwise rigid and brittle thallus to bend at these points, resulting in flexibility that allows coralline algae to grow up to 30 cm tall (Bold and Wyne 1985). Growth in coralline algae can be apical and intercalary, however since their cell walls are heavily impregnated with calcium carbonate they are slow growers (Johansen 1981). Due to allocation of energy and resources to structural components, coralline algae reduce their potential for photosynthesis and hence are slow growers and one of the lowest producers among macroalgae (Littler and Arnold 1982).

Numerous recent studies have focused on the effects of ocean acidification, caused by increased carbon dioxide concentration in the atmosphere, on marine species. Ocean acidification may harm marine calcifying organisms by reducing the rate of calcification of their skeletons or shells (Gao et al. 1993). As calcification in corallines is linked to photosynthetic carbon uptake, the carbon concentration in the atmosphere influences coralline algae. This in turn affects their photosynthesis and growth (Gao and Zheng 2010). As coralline algae are such important components of the marine ecosystem it is crucial to understand influences on their production and their growth rate in general. A few attempts have been made to quantify coralline

growth rate using various methods (Table 1), however since they are slow growers it is difficult to establish accurate results.

Table 1: Growth rates of articulate coralline, the methods used to obtain them, and the condition they were grow in (either culture or *in situ)*

Species	Growth rate	Method and growth condition	Author and Year
Corallina officinalis	0.066-0.068 mm day^{-1}	grid 1mm, culture	Colhart and Johansen 1973
Corallina officinalis	0.023-0.046 mm day^{-1} (*in situ)* 0.0232 mm day $^{-1}$ (culture)	alizarin red dye, culture & *in situ*	Andrake and Johansen 1980
Calliathron tuberculosum	0.083-0.133 mm day^{-1}	grid 1cm, *in situ*	Johansen and Austin 1970
Calliarthron cheilosporiodes	0.077-0.11 mm day^{-1}	Calcofluor white, *in situ*	Martone 2010
Corallina sessilis	2.1% day^{-1}	change in mass, culture	Gao and Zheng 2010

Calcofluor white is an optical brightener that can be used to stain cell walls of plants (Hughes and McCully 1975). Optical brighteners are colourless dyes widely used as whitening agents and are effective because they bind to a variety of materials, in particular textiles and papers. They fluoresce intensely in the blue range when exposed to long wavelength UV and short wavelength visible light. The stained plant walls are brilliantly fluorescent while most cytoplasmic components and unstained walls do not fluoresce. These brighteners have low toxicity with studies suggesting them not to be toxic to higher plant cells at all (Hughes and McCully 1975). Not only have fluorescent brighteners been used for the study of microorganisms (Darken 1962) but furthermore to analyse cell elongation in non-coralline red algae (Waaland and Waaland 1975) and quantify growth in corallines (Martone 2010). Martone (2010) used 0.02% solution of Calcofluor white to stain *Calliarthron cheilosporioides* in the field (Table 1), and analysed them up to 573 days after staining. This proved to be a useful and accurate method for measuring coralline growth rate.

Articulated (geniculate) coralline algae dominate many south and east coast shores of South Africa, and can be locally common on the west coast. Growth rate, either in culture or *in situ*, has never been measured in South Africa before. Hence, growth of geniculate corallines in South Africa is unknown.

A study was undertaken to test the use of Calcofluor white for measuring growth of local corallines. Two intertidal articulated coralline species were chosen as study subjects, the globally distributed *Corallina officinalis* and the South African endemic *Arthrocardia corymbosa.* The aims of the study were:

1. To test whether Calcofluor white could be used to measure growth in these species in culture and *in situ*
2. To compare growth rates of two intertidal coralline species grown in culture
3. To establish whether the time of exposure to the stain effects growth
4. To test whether the Calcofluor white staining technique can be used in a growth experiment in culture (effects of salinity on growth)
5. To establish whether the stain is visible after thalli have been preserved by 3 common methods, so that growth experiments can be done *in situ* in remote areas and thalli returned to the laboratory for measurements

Material and Methods

Culture vs. Nature

Corallina officinalis and *Arthrocardia corymbosa* were collected at Glencairn, South Africa (34° 09'44 S, 18° 25' 55 E). Tufts averaging around 5 cm were carefully removed from the rock near their base and brought back to the lab. Here they were rinsed in filtered seawater in an attempt to rid them of epiphytes and other organisms.

At the collection site *Corallina officinalis* fronds were stained in a rocky-tidal pool during low tide. Exposed fronds were dipped into a 0.02% Calcofluor white solution held in a plastic vial. The fluorescent dye solution was made up in 50 ml 2μm-filtered seawater and 0.01g Calcofluor white powder making solution of 0.02% (Martone 2010). The fronds were supposed to be submersed for at least 5 min. However, once dipped into the solution the seaweed tufts sucked up the liquid not allowing for long exposure. Stained fronds were tagged and removed from the rock after one month for analysis in the laboratory. Here they were viewed under a dissecting microscope magnified 16x while illuminated with a UV lamp.

Species Comparison and Exposure Time to the Fluorescent Dye

Five plastic culture bags were prepared for growing algae in the laboratory. Each bag contained 2 L of 2µm-filtered seawater and 40 ml Provasoli enriched seawater (PES) solution (Table 2, 3 and 4, Anderson 2005). In each bag a total of ± 9 g of plant mass, with similarly sized fronds, was placed. Prior to this plants were stained with 0.02% solution of Calcofluor white. Plants from bag 1 were stained for 15 min, plants from bag 2 for 30 min and plants from bag 3 for 5 min. Bags 4 and 5 served as controls.

Table 2: Provasoli enriched seawater (PES) solution

Ingredient	amount
$NaNO_3$	28 g
Na_2 glycerophosphate	0.4 g
Thiamine dichloride	0.004 g
Tris buffer	4 g
Vitamin B_{12} ($1mg.10ml^{-1}$)	0.8 g
Biotin ($1mg.20ml^{-1}$)	0.8 g
Fe EDTA solution*	200 ml
P_{II} solution*	200 ml

*Table 3 and 4

Table 3: Fe EDTA solution

Ingredient	amount
$Fe(NH_4)_2(SO_4)_2.H_2O$	0.07 g
Na_2 EDTA	0.6 g
Distilled water	1000 ml

Table 4: P_{II} solution

Ingredient	amount
H_3BO_3	1.14 g
$FeCl_3.6H_2O$	0.049 g
$MnSO_4.H_2O$	0.164 g
$ZnSO_4.7H_2O$	0.022 g
Na_2 EDTA	1 g
$CoSO_4.7H_2O$	0.005 g
Distilled water	1000 ml

The bags were hung in a 15°C constant temperature room with light:dark period set to 16 hours daylight:8 hours dark. Each bag was aerated via an electromagnetic air compressor (Boyu AQ-003, 35 W, 220 V), which also kept water in motion and nutrients circulating. The medium was changed and pH measured once a week. Algae were grown for a total of 28 days after which epiphytes smothered the corallines. Five

to seven fronds of each species were removed after 8, 20 and 28 days to be photographed for measurements. Stained fronds were placed under a dissecting microscope and illuminated with an ultraviolet lamp, magnified 16x and photographed digitally (Canon Inc, G7 camera) with long exposure (1sec). Growth rate was quantified by measuring the length of newly deposited tissue distal to fluorescent chemical marks and dividing by the time elapsed since stain application. Three to six randomly selected tips were measured in each frond to yield an average growth rate per frond. Each bag therefore provided a sample size of 5-7 averages, which were statistically compared using STATISTICA 9 (StatSoft Inc). Kruskal-Wallis ANOVA test, followed by a post-hoc test was used to test for significance.

Effects of Salinity on Growth

Nine culture bags were prepared for growing *Corallina officinalis* under three different salinity conditions in the same culture room as mentioned above. These conditions were 100% seawater (±35 ppt salinity), 60% seawater (± 21 ppt salinity) and 30% seawater (± 10 ppt salinity). Bag 1, 2 and 3 contained 2 L of 2μm-filtered seawater and 40 ml PES solution. Bag 4, 5 and 6 contained 1.2 L of 2μm-filtered seawater, 0.8 L distilled freshwater and 40 ml PES solution. Bags 7, 8 and 9 contained 0.6 L 2μm-filtered seawater, 1.4 L distilled freshwater and 40 ml PES solution. Weights of plants were taken before and after with initial average weight being 4-6 g. The algae were stained in 0.02% Calcofluor white solution for 15 min. After 11 days 5-6 fronds were selected from each bag, magnified 16x and whilst illuminated with UV-light photographed with long exposure (1sec). On each frond 3-5 tips were measured to obtain an average growth rate.

Preservation Method and Stain visibility

Corallina officinalis fronds that were stained with the fluorescent dye were then preserved in one of three ways; by drying, or in 5% formalin in seawater, or in 70% alcohol/ 20% glycerol/ 10% freshwater solution. These are methods used by seaweed biologists to preserve seaweeds for later analysis. Seaweeds preserved in formalin are used for later sectioning or microscopic observation. Dried seaweed can be used for DNA extraction, whereas the alcohol and glycerol solution is a special preservative used for coralline algae DNA extraction. After 9 days each frond was illuminated

with a UV-light, magnified 16x and photographed under a dissecting microscope with long exposure (1sec) to detect if fluorescent marks were still visible.

Results

Culture vs. Nature

There were no visible fluorescent marks on *Corallina officinalis* stained *in situ*, when the thalli were recovered and brought back to the laboratory 28 days after staining. Because the staining failed all further analysis was carried out on corallines grown in culture.

Species Comparison

The pH in all bags stayed at 7-8 throughout the experiment. After 8 and 20 days there was no visible growth by *Arthrocardia corymbosa* (Figure 1). After 28 days some of the tufts did not show growth (Figure 1c), whereas some showed growth (Figure 1d). However, too few tufts had grown to calculate growth rates and further experiments with this species were discontinued. *Corallina officinalis* growth was visible at day 8, 20 and 28 with clear white bands where initial tissue was stained (Figure 2).

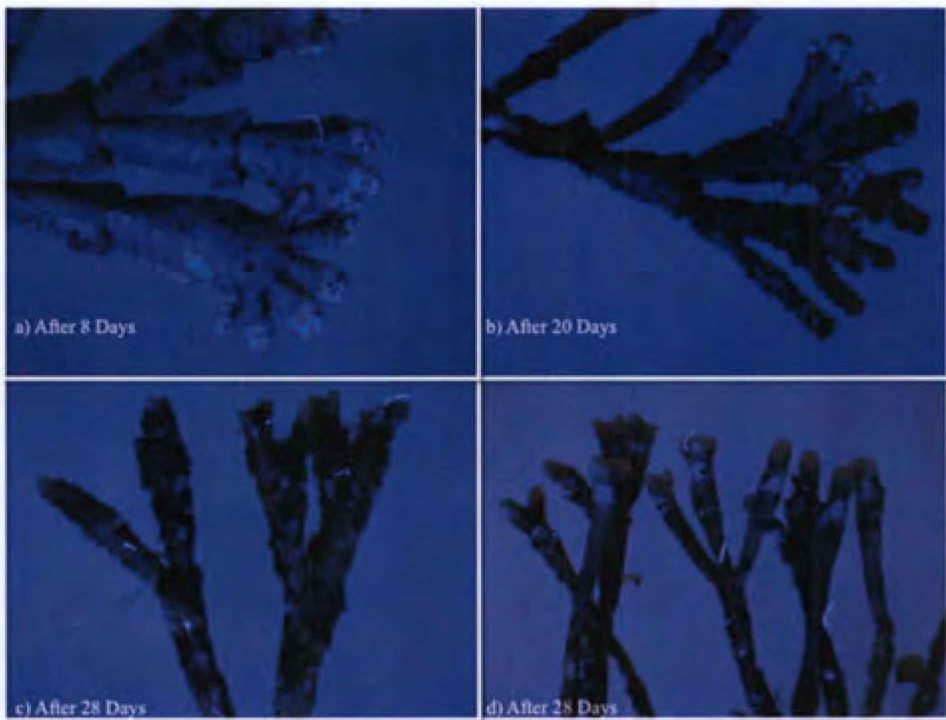

Figure 1: Examples of *Arthrocardia corymbosa* after 8, 20 and 28 days, where only sample d) showed growth

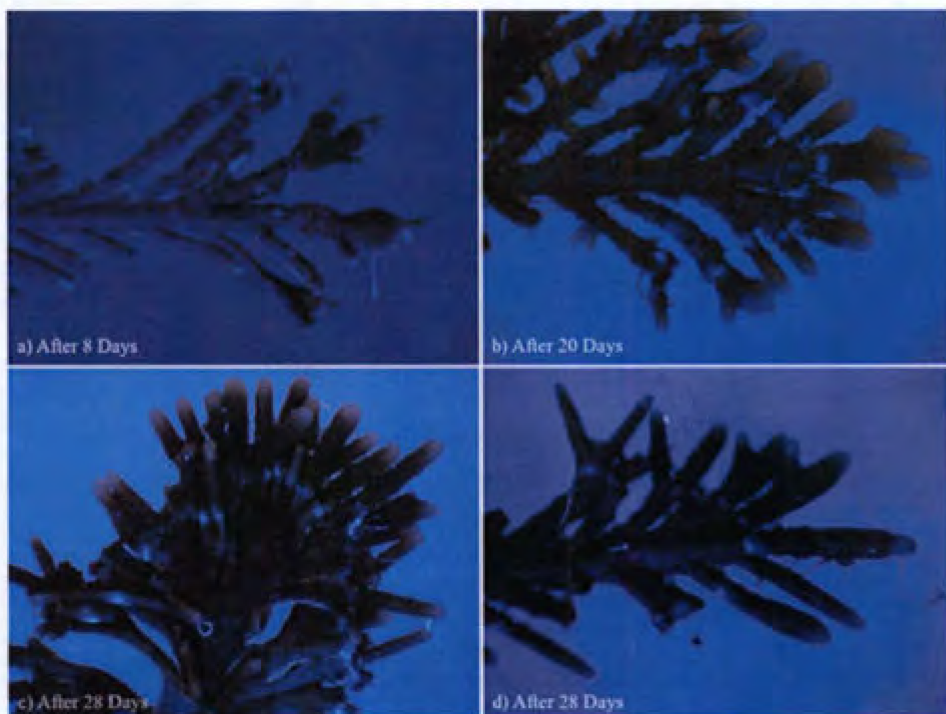

Figure 2: Examples of *Corallina officinalis* after 8, 20 and 28 days, with a clear white band as the fluorescent mark.

Exposure Time to Fluorescent Dye

Corallina officinalis showed a clear white band that marks the area of initial staining, with new tissue growth distal to that white band. An example of the measuring growth procedure is depicted in Figure 3. This method was used for all further growth rate measurements of *Corallina officinalis*.

All thalli, whether stained for 5, 15 or 30 min, grew in culture and showed measurable bands after 8, 20 and 28 days (Figure 4, 5 and 6, respectively). After 20 and 28 days, plants that had been stained for 15 min had grown significantly slower than those stained for 5 and 30 min ($p<0.05$). Overall average of all three exposure times, *Corallina officinalis* grew at a rate of 0.08 mm day $^{-1}$.

Figure 3: Arrow pointing at fluorescent stained band, with growth of new tips behind fluorescent mark. Tips 1-5 were measured and averaged to obtain growth rate per frond.

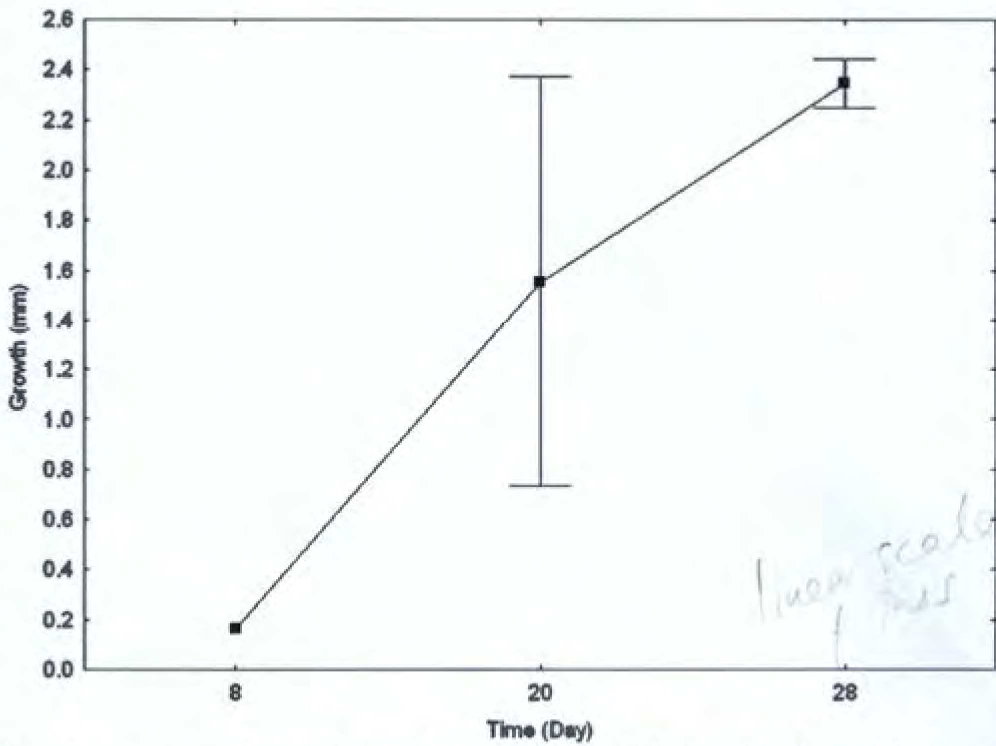

Figure 4: Average growth rate ± SE (mm/day) of *Corallina officinalis* stained for 5 min in fluorescent dye and grown in culture for 28 days.

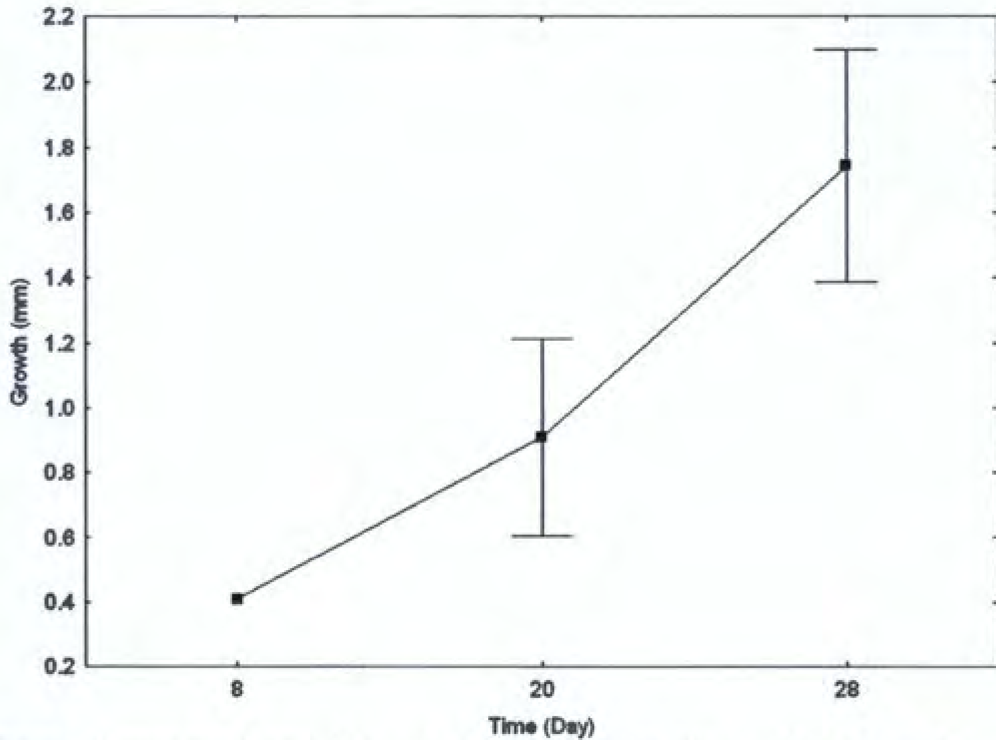

Figure 5: Average growth rate ± SE (mm/day) of *Corallina officinalis* stained for 15 min in fluorescent dye and grown in culture for 28 days.

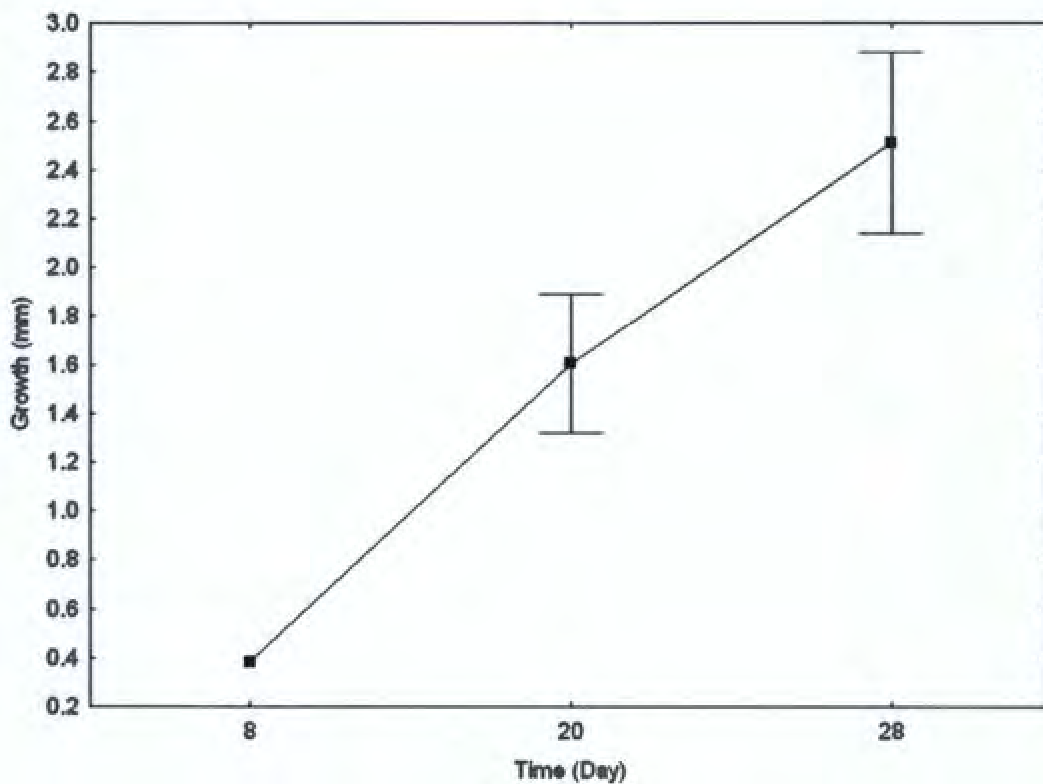

Figure 6: Average growth rate ± SE (mm/day) of *Corallina officinalis* stained for 30 min in fluorescent dye and grown in culture for 28 days.

Effects of Salinity on Growth

Corallina officinalis plants that were kept in full seawater salinity conditions grew 1.03 mm in 11 days, which equates to 0.09 mm per day (Figure 7). Plants kept in 60% seawater (21 ppt) grew 0.37 mm in 11 days, which equates to 0.03 mm per day (Figure 7). Plants growing in 30% seawater (10 ppt) showed no growth (Figure 7). Differences between treatments were so marked that statistical tests were unnecessary.

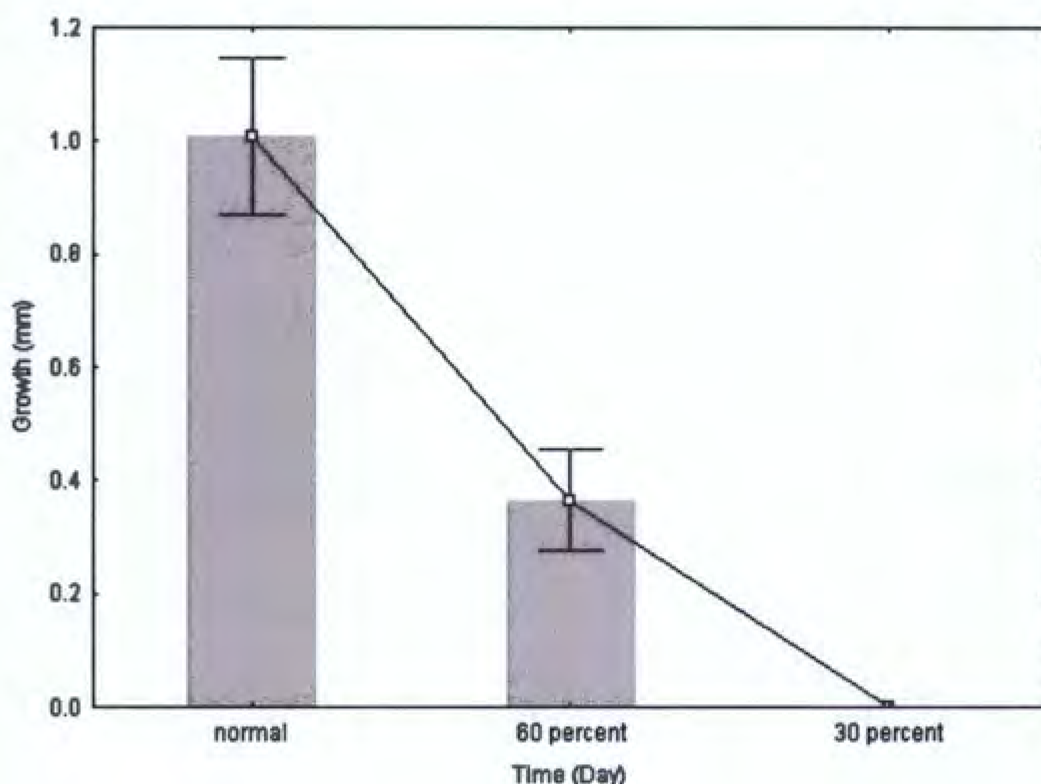

Figure 7: Actual growth ± SE (mm) in 11 days of *Corallina officinalis* under three salinity treatments.

Preservation Method and Stain visibility

After two weeks, the fluorescent mark was visible on fronds of *Corallina officinalis* preserved in formalin or dried (Table 2). The mark on fronds which were preserved in the alcohol-glycerol solution were highly visible on fronds stained for 30 min but could only be seen vaguely on fronds stained for 5 and 15 min (Table 2).

Table 2: Stain visibility after preservation of corallines stained for 5, 15 and 30 min in fluorescent dye

Staining time	Alcohol-Glycerol	Formalin	dried
5 min	no/very vague	yes	yes
15 min	very vague	yes	yes
30 min	yes	yes	yes

Discussion

Culture vs. Nature

The attempt to stain *Corallina officinalis in situ* failed, possibly because the fronds were not exposed to the stain for long enough. To stain coralline algae in the field a careful method needs to be designed to ensure the algae are exposed for a minimum of 5 min to the fluorescent dye. Martone (2010) successfully stained *Calliarthron cheilosporioides* in the field by submersing fronds for 5 min in a 0.02 % Calcofluor white solution. However, in comparison *Calliarthron cheilosporioides* has longer fronds up to 15 cm (Martone 2010), whereas *Corallina officinalis* longest frond is only 5 cm long. *Corallina officinalis* fronds could not be immersed individually without the whole thalli being dunked in the Calcofluor solution. Since the base of the thalli is dry, the fluid is sucked up, not allowing fronds to be immersed for long. Therefore, for small/short algae I suggest a "bag method" where a small water resistant bag is tied around the algae and the calculated fluorescent dye injected into the bag. This method would also allow for staining of subtidal species. Algae grown in culture can easily be stained due to them not being attached to a substrate. Therefore using the dye in the lab is easy and efficient.

Species Comparison

Arthrocardia corymbosa either grows very slowly or does not grow well under culture conditions. Their growth rate was too slow to detect and measure within 28 days and growing them much longer is not possible due to epiphyte cover. *Corallina officinalis* in contrast proved to be a relatively fast grower under lab conditions. Growth was visible even after 8 days, making this species viable for experimenting with the fluorescent dye. Why these two species grow at different rates is not conclusive as the culture conditions were identical. However, their distribution varies with *Arthrocardia corymbosa* growing on the sublittoral fringe and below and *Corallina officinalis* found in the lower intertidal (Stegenga *et al.* 1997). *Corallina officinalis* being more subjected to an unstable wave-swept environment may have developed higher tolerance and faster growth due to higher breakage of their fronds. This resulting in *Corallina officinalis* growing faster under favourable culture conditions.

Exposure Time to Fluorescent Dye

A clear white fluorescent band equating to the time of staining was visible on corallines exposed to the stain. Stain visibility does not vary between plants stained for 15 min to those stained for 30 min. *Corallina officinalis* exposed to the stain for 5 min showed a slightly fainter fluorescent band, yet was still visible and could be used for measuring growth. This finding is important for using the stain in the field where time may be limiting. The fluorescent dye did not inhibit growth of *Corallina officinalis* as all samples exposed to the dye grew. It was difficult to compare growth of the stained samples to that of unstained samples (control) as taking the corallines weight was inaccurate due to factors such as water still left on plants and broken off tips. Taking weights of the coralline before and after proved to be inaccurate due to these constraints. Growth was significantly different between plants exposed to the dye for 15 min to those exposed for 30 min. Since there was not a significant difference between plants exposed for 5 min to those exposed for 30 min suggests that the exposure time to the dye is not the cause of this growth difference. Instead, other factors such as the circulating, or aerating of the medium and position towards the light could play a role. Visually all bags were aerated similarly and they were equally positioned regarding the light, however since aeration is not measurable a concrete conclusion cannot be drawn. However, to draw a concrete conclusion the experiment needs to be repeated with at least three replicates (bags) of each exposure time so that all other factors can be eliminated and the only variance being exposure time to the dye. On average, *Corallina officinalis* of Bags 1, 2 and 3 grew 0.08 mm day $^{-1}$, which is comparable to a growth rate of 0.066-0.068 mm day $^{-1}$ of *Corallina officinalis* grown in culture by Colhart and Johansen (1973).

Effects of Salinity on Growth

Corallina officinalis grown under normal conditions had a growth rate of 0.09 mm day $^{-1}$, which is comparable to the growth rate of 0.08 mm day $^{-1}$ from the experiment on exposure time to the dye and to the study done by Colhart and Johansen (1973) who measured a growth rate of 0.066-0.068 mm day $^{-1}$. The growth rate of 0.09 mm day $^{-1}$ is higher than growth rate of 0.023- 0.047 mm day $^{-1}$ of *Corallina officinalis in situ* and 0.023 mm day $^{-1}$ in culture measured by Andrake and Johansen (1980). *Corallina officinalis* grown in 60 % saltwater (±21 ppt) grew very slowly and did not

grow at all in 30% saltwater (±10 ppt). The coralline algae subjected to 30 % saltwater (±10 ppt) showed a clear bright fluorescent mark at each tip of all fronds, showing that the coralline algae were successfully stained but never grew. As *Corallina officinalis* is an intertidal species we expect it to tolerate salinity fluctuations to a certain extend. Because fresh water floats on salt water, salinity will increase little in mid-intertidal and low-intertidal pools except following torrential downpours. However, when these downpours occur and the salinity in a rock pool decreases algae occurring in these will have evolved tolerances to survive the temporary change of their environment. Diluted seawater causes a decline in photosynthesis and hence a decline in metabolic rate in several marine plants (Lobban and Harrison 1994). Lowered salinities often stunt the growth of seaweeds and have variable effects on branching (Norton *et al.* 1981 in Lobban and Harrison 1994) mainly by the loss of ions or inhibition of enzymes (Lobban and Harrison). However, the exact physiological response of *Corallina officinalis* and other geniculate corallines to decreased salinity is still largely unknown. What can be deduced is how this simple ecophysiological experiment showed that fluorescent dye could be used to measure growth in coralline algae in laboratory experiments. This method can be adopted for growth measurements that are difficult to obtain, for example species that grow to slow to be weighed can instead be dyed and measured.

Algae Preservation

The alcohol in the alcohol/glycerol solution could act as an anti-staining agent, which slowly dissolves the fluorescent stain, similarly how the alcohol dissolves plant pigment such as chlorophyll leaving the thallus white. Preserving algae in alcohol/glycerol solution of longer than one week is therefore not recommended. Formalin and drying does not affect the staining and can be used to preserve algae that have been stained for min 5 min and are to be analysed in the future. This is especially useful when wanting to measure algae growth in remote places. As staining last over one year (Martone 2010), it is possible to stain algae in the field leave them for extended time and return for collection. Samples can be dried or preserved in formalin for over one week and analysed once returned to the laboratory.

Conclusion

Calcofluor white is an effective fluorescent dye that can be used to stain coralline algae. A clear fluorescent-white band corresponded to the position of apical meristems at the time of stain application on *Corallina officinalis*. All new apical growth could be seen and measured, with a dissecting microscope and illuminated with a UV-lamp, behind the fluorescent band. Similarly how Gao and Zheng (2010) analysed the response of a geniculate coralline algae to ocean acidification and solar radiation by growing them in culture can Calcofluor white be used to measure algae growth rate. Laboratory experiments such as adjusting pH, carbon and temperature can be set up and geniculate coralline algae growth easily measured by the use of the fluorescent dye. This method of measuring growth rate can prove to be useful especially to expand our understanding how global changes, such as increased atmospheric carbon affects coralline algae growth and therefore entire marine ecosystems.

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