

Effects of initial enrichment of nitrogen and phosphorus on *Bostrychia* and *Caloglossa* (Ceramiales, Rhodophyta) growth using digital imaging

Kelly Ryder,¹ John West* and David Nicholls²

¹School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia and ²School of Biological Sciences, University of New South Wales, Sydney, New South Wales 2052, Australia

SUMMARY

Growth experiments measured the effects of an initial starvation period followed by a single nutrient pulse. Nutrient pulses were conducted at four different N-levels (65, 1514, 2900, and 6080 $\mu\text{g N L}^{-1}$) and four different P-levels (84, 281, 639, and 849 $\mu\text{g P L}^{-1}$) using isolates of *Bostrychia moritziana* (Sonder ex Kützing) J. Agardh and *Caloglossa leprieurii* (Montagne) G. Martens. Specific growth rates (% day⁻¹) in primary axis length (*B. moritziana* only) and surface area were measured using digital imaging. Results showed that the specific growth rates of both algae were highly dependent on the N-levels of the seawater ($P < 0.001$ – 0.05). Specific growth rates for the *B. moritziana* were approximately 3.5% day⁻¹ at $t = 7$ days declining to approximately 1.0–1.5% day⁻¹ at $t = 49$ days for the primary axis length, and approximately 8–9% day⁻¹ declining to approximately 2–3% day⁻¹ for surface area. The specific growth rate declined more rapidly with decreasing N initially added to the medium. In *C. leprieurii*, the specific growth rates for all four conditions at $t = 8$ days were approximately 11–13% day⁻¹, but N/O declined to approximately 3% day⁻¹ whereas the others declined to only approximately 7–8% day⁻¹ by $t = 49$ days. The effects of initial P-levels on growth varied, but generally indicated a direct relationship with specific growth rate. In *C. leprieurii*, the number of nodes and blades per plant was also measured via digital imaging and were found to increase with increasing N-levels, whereas P-levels had no influence. It was concluded that *B. moritziana* and *C. leprieurii* are prone to N-limitation, but P-limitation is less prone. Using digital imaging to measure the specific growth rate in total surface area and primary axis length provided a significantly more accurate depiction of the rate of growth than some of the more conventional means of measuring growth rate.

Key words: *Bostrychia*, *Caloglossa*, digital imaging, growth, mangroves, nitrogen, phosphorus, red algae.

INTRODUCTION

Algae associated with mangrove environments (e.g. *Bostrychia*, *Caloglossa*, *Catenella*) are subjected to extreme fluctuations in nutrient availability. The algae generally grow intertidally on the trunks and roots of mangrove trees, as well as on the sediment (Edyvane 1995). This habitat restricts their nutrient exposure to that brought in with the tide or carried in with freshwater inputs such as river discharge, rainfall, and land drainage (Hutchings and Saenger 1987). In mangrove ecosystems, and much of the marine environment, nitrogen and phosphorus are generally regarded as the most limiting nutrients to algae (Ryther and Dunstan 1971; Gordon *et al.* 1981; Clough *et al.* 1983).

The available literature on nutrient levels in the mangrove environment mainly centers around: (i) terrestrial carbon levels as a result of decomposing detritus (Edyvane 1995; Kristensen *et al.* 1995); (ii) lists of the major sources of nutrient input/output to the mangrove ecosystem as a whole (Hutchings and Saenger 1987); and (iii) soil and leaf nutrient levels, such as N and P (Lugo *et al.* 1976; Onuf *et al.* 1977; Boto and Wellington 1983; Hutchings and Saenger 1987; Feller 1995). Because these components of the mangrove ecosystem are interconnected with the marine aspect, inferences can at best only be drawn to give some indication of the nutrient levels available to the algae.

Literature on nutrient levels present in the mangrove marine environment is seriously lacking. Edyvane (1995) reports that mangrove debris in Victoria, Australia, rapidly breaks down in the coastal waters, releasing nutrients (i.e. N and P), which seem to become available at least to the terrestrial plant life. A mangrove system near Adelaide, South Australia, suffered large-scale growth of micro- and macroalgae after having been exposed to sewage discharge and stormwater and the resultant increase in nutrients (i.e. N and P) (Edyvane 1995). Studies by Boto (1982) of an Australian mangrove system showed

* To whom correspondence should be addressed.

Email: <j.west@rubens.its.unimelb.edu.au>

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low nutrient levels in tidal creek waters with the probable main influx of nutrients via tide-borne particles. Despite this minimal amount of information available, knowledge of the nutrient levels present in mangrove waters is essential in defining the limits of algal productivity.

Nutrients present in the water column can be found in a wide variety of forms, and not all are biologically available. Although nitrate, nitrite, ammonia and urea are all potential sources of nitrogen in the water column, nitrate (NO_3) and ammonia (i.e. NH_3 plus NH_4) provide the two forms of inorganic nitrogen most important to red algae (Parsons and Harrison 1983). Phosphorus exists in seawater as inorganic orthophosphate, metallophosphate complexes, polyphosphates and organic phosphorus compounds (Cembella *et al.* 1983), but is principally acquired by marine algae as free orthophosphate (Raven 1980).

A number of factors affect the nutrient levels present in algae. The rate of nutrient uptake is subject to changes due to thallus surface area (Wallentinus 1984), water chemistry (Caperon and Meyer 1972; Hanisak and Harlin 1978; Lapointe and Tenore 1981; Lapointe *et al.* 1984), light intensity (Lewin 1962), and culture preconditioning (Cembella *et al.* 1983). Nutrient availability is influenced by factors such as water movement (Kain and Norton 1990), seasonal fluctuations in nutrient concentration (Kornfeldt 1982; Wheeler and Bjornsater 1992), and light (Haines and Wheeler 1978; Lapointe 1981; Lapointe and Tenore 1981; Lapointe and Duke 1984).

Furthermore various algae are capable of storing nutrients (ammonia, nitrate, nitrite, phosphate, ammonium) during the winter when their supply is in excess of need, and then utilizing these stores in order to extend the growing period into the nutrient-limited summer months (Chapman and Craigie 1977; Laycock and Craigie 1977; Hanisak 1979; Gordon *et al.* 1981; Kornfeldt 1982; Rosenberg and Ramus 1982; Lapointe and Duke 1984; Rosenberg *et al.* 1984; Fujita 1985). Nitrogen reserves can be found as protein complexes, soluble compounds such as nitrate and amino acids, and phycobillin pigments (Laycock and Craigie 1977; Bird *et al.* 1982; Wyman *et al.* 1985). The compounds that serve as a nitrogen pool vary from species to species, as does the ability of algae to store nitrogen (Fredriksen and Rueness 1989). Excess phosphate can be stored as vacuolar phosphate (Cembella *et al.* 1983) or as cytoplasmic polyphosphate granules (Kuhl 1962). Further information does not seem to be available on phosphate reserves in macroalgae.

In the last few years, research has been carried out on aspects of the ecophysiology, osmotic acclimation and the polyol cycle of the mangrove algae *Bostrychia* and *Caloglossa* (Karsten and West 1993; Karsten *et al.* 1993; Mostaert and King 1993; Mostaert *et al.* 1995a,b; Karsten *et al.* 1996; Mostaert *et al.* 1996; Karsten *et al.* 1997a,b), but as yet no research has been performed on the effects of nutrient levels on mangrove algae. In order to expand the available infor-

mation on this topic, the effects of four different phosphate and nitrate levels on the growth of *Bostrychia moritziana* (Sonder *ex* Kützing) J. Agardh and *Caloglossa lepriurii* (Montagne) G. Martens were studied.

With recent technological advances in digital imaging and the present, widespread use of this technology in many science fields (e.g. palaeontology, fisheries biology, histology, developmental biology, comparative physiology, neurology), we decided to test its usefulness at measuring the growth of small, complex macroalgae. In the past, researchers have used various means of measuring the growth of algae such as: fresh weight (DeBoer *et al.* 1978; Gordon *et al.* 1981; Sousa-Pinto *et al.* 1996), length of the laterals (Karsten and West 1993; Karsten *et al.* 1993) and/or the main axis, and combinations thereof. In 1993, Gonzalez and Goff used a form of digital imaging to measure *Microcladia* sp. germlings, but very little information was provided on the technique or its results.

Since algae such as *Bostrychia* and *Caloglossa* sp. grow three-dimensionally with a great deal of latitudinal growth, the above-mentioned conventional methods of measuring growth (in particular, surface area) would be inadequate. Digital imaging provides two-dimensional arrays of pixels (picture elements) to represent the video camera images. According to the image intensity of a local area, the representative pixels are assigned one of a range of integers which corresponds to a shade of black, white or gray. The video camera image is reconstructed on the computer where it can be distinguished from the background. Image processing functions can augment the image quality through such things as contrast enhancement, density profiling, smoothing, sharpening, background subtraction and edge detection. Thus, the exact outline of the alga with all its branching can be obtained and various features can be determined such as surface area and length of a selected area, among others (information taken from the National Institute of Health Image web site; see Materials and Methods section for web address).

MATERIALS AND METHODS

Stock cultures

Two isolates of *B. moritziana* and two isolates of *C. lepriurii* were used in these experiments (Table 1). The methods for isolation into culture were as outlined in West and Calumpong (1988). The seawater for all culture work was obtained from Williamstown, Port Phillip Bay, Victoria. The stock cultures were maintained in Pyrex® no. 3250 (Corning Glass Works, Corning, NY, USA) 100 × 80 mm storage dishes with 200 mL Provasoli's enriched seawater (30‰) under low light (approximately 5–6 $\mu\text{mol photons m}^{-2}$ per s) supplied by cool-white fluorescent lights and a 12:12 L:D photoperiod cycle. The ambient temperature of the culture room was maintained at $25 \pm 2^\circ\text{C}$.

Experimental procedure

Female plants were used in the present study because they grew well in culture and unlike the sporophytic or male phases, the females did not release reproductive products that would have interfered with growth measurements. For the *Bostrychia* specimens, excised apical portions (approximately 1 cm) were placed in unenriched, sterile seawater (in a 100 × 80 mm glass dish) for 7 days prior to the start of the experiment in order to reduce any possible internal stores of nutrients (Lewis and Hanisak 1996). The *Caloglossa* specimens consisted of an apical blade excised approximately two-thirds down from the upper node, with two small laterals at the remaining node. These cuttings were also 'starved' for 7 days before the start of the experiment. All plants were kept under identical conditions as the original stocks, except the cuttings were randomly placed each week on rotary shakers (75 r.p.m.) supplied with 6–17 μmol photons m⁻² per s. Irradiance levels were regulated using gray neutral density plastic window-screening with 1 mm mesh size. All irradiance measurements were made with a Li-Cor Model LI 189 (Li-Cor Inc., Lincoln, NB, USA) integrating quantum/radiometer/photometer.

After the 7 day starvation period, the cuttings were transferred to 25 mL of variously enriched sterile seawater in either Pyrex® (Corning Glass Works, Corning, NY, USA) 50 × 35 mm crystallizing dishes covered with 50 mm Petri dish lids, or Pyrex® no. 3140 70 × 50 mm crystallizing dishes covered with Glad® Cling Wrap (Glad Products, Padstow, NSW, Australia) clear polyethylene and an elastic band. The polyethylene wrap reduced evaporation and prevented salinity increases. Provasoli's enrichment (Starr and Zeikus 1993) was initially made without the nitrate or phosphate added (Table 2). Additions of N (as NaNO₃) and P (as KH₂PO₄) were made as shown in Table 3. One branch was added to each of the five replicate dishes for the eight conditions. The cuttings were otherwise kept under the same conditions as listed above for the 7 day starvation period. Two identical experiments were carried out using *Bostrychia* 2748, one experiment using *Bostrychia* 3571, and one using *Caloglossa* 3543.

In order to more closely control the nutrient levels, an attempt was made to grow *B. moritziana* 2748 and *C. lepriurii* 3352 in marine culture medium artificial seawater (Woelkerling *et al.* 1983).

Growth measurements

Using a digital imaging camera, the growth of each *Bostrychia* cutting was measured by the increase in its primary axis length and in the plant's overall surface area; while for *Caloglossa* cuttings, the surface area, the number of nodes per plant, and the number of blades per plant were measured. Wet-mounts were made with the specimens using a few drops of the growth medium.

For the short time between slide preparation and imaging, the slides were kept in a plastic tray tightly covered with aluminum foil to reduce bubble formation by photosynthesis and avoid errors in surface area measurements. The initial measurements were taken at the time of transfer to the enriched seawater and were recorded as t = 0 days. Every 7 days from t = 0, subsequent measurements were made of the cuttings.

Specific growth rate (SGR)(% day⁻¹) was determined using the formula described in Karsten *et al.* (1993):

$$\text{SGR} = 100 (\ln L_t - \ln L_i)t^{-1}$$

where t is the time in days, L_t is the length at time t (starting at t = 7 days), and L_i is the initial length at t = 0 (L_t and L_i were substituted by the surface area measurements, SA_t and SA_i, respectively, where appropriate). Specific growth rates of primary axis length and surface area were computed for the five replicates in each set of eight conditions. The five replicate measurements per condition (SA, L, number of nodes and

Table 1. Culture isolates used for experiments

Species, culture no.	Collection site and date
<i>Bostrychia moritziana</i> 2748 tetrasporophyte	Tooradin, Victoria, Australia, 25 October 1986
<i>Bostrychia moritziana</i> 3571 tetrasporophyte	Stuarts Point, New South Wales, Australia, 22 October 1995
<i>Caloglossa lepriurii</i> 3352 tetrasporophyte	Fullerton Cove, New South Wales, Australia, 12 March 1993
<i>Caloglossa lepriurii</i> 3543 tetrasporophyte	Hastings Point, New South Wales, Australia, 23 October 1995

Table 2. Provasoli's stock solution made without nitrogen or phosphorus

Ingredients	Method
200 mL EDTA stock	Bring to 1000 mL with Milli-Q water
200 mL PII stock	Add 100 mL of the above to each
8 mL Thiamine stock	of the enrichments
8 mL Biotin stock	
3.2 mL B12 stock	

Table 3. Enrichments to Provasoli's stock (phosphorus as KH₂PO₄, nitrogen as NaNO₃)

	Phosphorus (25 g L ⁻¹)	Nitrogen (35 g L ⁻¹)
N/0	0.8 mL	None
N/4	0.8 mL	2 mL
N/2	0.8 mL	4 mL
N/1	0.8 mL	8 mL
P/0	None	8 mL
P/4	0.2 mL	8 mL
P/2	0.4 mL	8 mL
P/1	0.8 mL	8 mL

blades) were averaged in order to graph their change over time.

To photograph the algae for later measurement, an enlarging table was used to which a Bolex Macro-Switar 16 mm movie camera lens (1:1.9, Zurich, Switzerland) and an Electrim black and white digital computer camera (EDC-1000U) were attached. A Schott KL1500 light source (Feldbach, Germany) affixed to a Zeiss stereomicroscope (Oberkochen, Germany) base was used to illuminate the specimens from below the working surface. Images were stored on Zip™ 100 MB disks (Imago Co., Roy, UT, USA) for processing.

During the course of the experiment (approximately 49 days per trial), the cuttings were also examined weekly with a Zeiss stereomicroscope. Notes were taken on changes in color, general appearance, cell morphology and/or structural differences.

Processing images

Digital Images were analyzed using a Macintosh Centris 650 computer (Macintosh, Cupertino, CA, USA) using the public domain National Institutes of Health Image program (available on the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI).

The cuttings were measured by density slicing the images. After setting the scale on an image file, the background was subtracted (selecting 2D rolling ball) and a section of the image showing fine detail was magnified. The image was density sliced at that level best representing the recorded image. In order to measure the surface area, the cutting was traced to exclude any unwanted dark spots or lines. For the *Bostrychia* cuttings, the primary axis length was measured by freehand-tracing its length. For the *Caloglossa* cuttings, the number of nodes per plant and the number of blades per plant were counted while the image was magnified.

The digital imaging technique was determined to be accurate and reliable by first conducting several trial runs where the primary axis length was measured with a ruler as well. Thereafter, one specimen per dish was measured in both manners as quality assurance. However, the following sources of potential error in measurement should be noted by anyone who will be

using the digital imaging technique described in this paper. When preparing slides for imaging, the overlap of branches or blades in organisms with morphologies similar to *Bostrychia* or *Caloglossa* species may create errors in accurately determining the surface area and also in counting the number of blades or nodes per plant. These flaws were recognized in our study and were found to cause only insignificant errors in estimating the total numbers present.

Data analysis

Standard errors were determined (± 1 SE). Statistical analysis involved a one-way ANOVA to compare the growth rates at each experiment's endpoint among the four nitrate concentrations and the four phosphate concentrations used in each trial.

Nutrient analysis

The seawater medium used in each dish was analyzed for phosphorus and nitrogen levels at the beginning and end of each trial. The medium from the five replicates for each nutrient level were pooled and frozen until needed. Initial water was derived by making an extra 200 mL of the seawater/media mixtures when the experiments were being prepared. The medium was pooled after the algae were removed on the final day. Levels of oxidized nitrogen in the seawater were determined using a modified cadmium reduction method (Major *et al.* 1972; American Public Health Association 1989). The levels of dissolved potassium dihydrogen phosphate in the seawater were determined using a modified single solution (ascorbic acid) method (Major *et al.* 1972; American Public Health Association 1989). Four subsamples were determined for each nutrient-level, the mean concentrations of which are given in Tables 4 and 5.

RESULTS

Specific growth rate over time using the primary axis length of *B. moritziana* 2748 was highly dependent on the nitrate concentration in the medium ($P < 0.001$) (Fig. 1). Similar results ($P < 0.05$) were found with *B. moritziana* 3571 (Fig. 3). In the phosphate series using *B. moritziana* 2748 and 3571 (Figs 2 and 4), all

Table 4. Initial and final nitrogen levels in the medium ($\mu\text{g N L}^{-1}$)

	Initial				Final			
	N/0	N/4	N/2	N/1	N/0	N/4	N/2	N/1
Axis length								
<i>Bostrychia moritziana</i> 2748	64.5	1514.2	2900.2	6080.0	< 10	< 10	< 10	223.3
<i>Bostrychia moritziana</i> 3571	64.5	1514.2	2900.2	6080.0	< 10	< 10	1090.7	3084.0
Surface area								
<i>Bostrychia moritziana</i> 2748	64.5	1514.2	2900.2	6080.0	< 10	< 10	< 10	< 10
<i>Caloglossa leprleurii</i> 3543	64.5	1514.2	2900.2	6080.0	< 10	< 10	< 10	506.4

Table 5. Initial and final phosphorus levels in the medium (in $\mu\text{g P L}^{-1}$)

	Initial				Final			
	P/0	P/4	P/2	P/1	P/0	P/4	P/2	P/1
Axis length								
<i>Bostrychia moritziana</i> 2748	83.8	280.9	639.2	848.8	< 10	< 10	167.5	402.9
<i>Bostrychia moritziana</i> 3571	83.8	280.9	639.2	848.8	13.8	80.8	356.5	467.8
Surface area								
<i>Bostrychia moritziana</i> 2748	83.8	280.9	639.2	848.8	< 10	< 10	129.3	310.6
<i>Caloglossa leprleurii</i> 3543	83.8	280.9	639.2	848.8	< 10	34.4	318.7	463.9

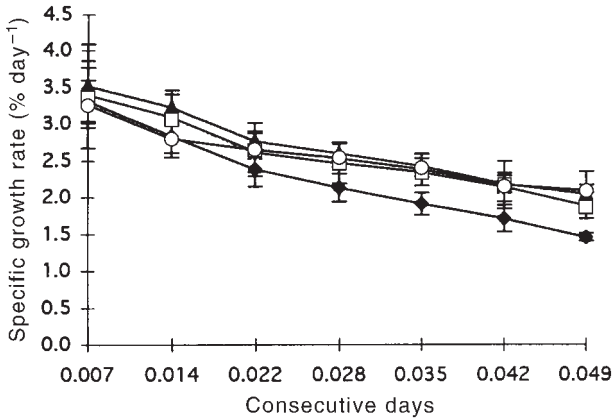


Fig. 1. Average specific growth rate in primary axis length of *Bostrychia moritziana* 2748 (female) after nitrogen enrichment: \blacklozenge , N/0; \square , N/4; \blacktriangle , N/2; \circ , N/1.

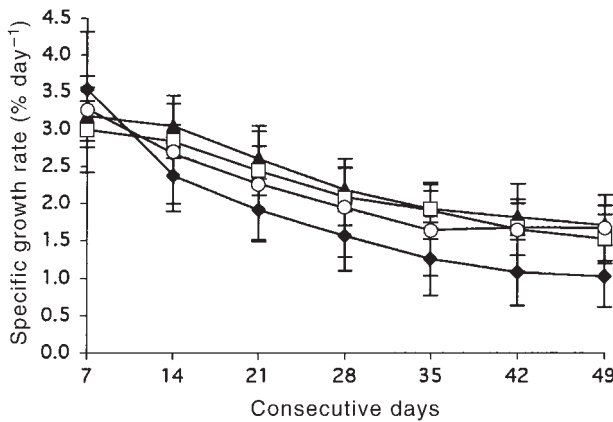


Fig. 2. Average specific growth rate in primary axis length of *Bostrychia moritziana* 3571 (female) after nitrogen enrichment: \blacklozenge , N/0; \square , N/4; \blacktriangle , N/2; \circ , N/1.

P-concentrations had the same effect on the growth rate in primary axis length ($P > 0.05$).

Trials using both *B. moritziana* 2748 and 3571 all showed very strong evidence ($P < 0.001$) that specific growth rate in surface area was highly dependent on the nitrate concentrations available in the ambient seawater (Figs 5, 7). In the P-series, both trials using *B. moritziana* 2748 showed that growth rate in surface area was signi-

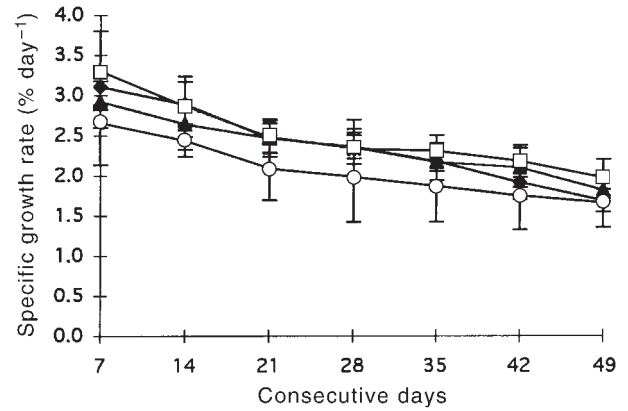


Fig. 3. Average specific growth rate in primary axis length of *Bostrychia moritziana* 2748 (female) after phosphorus enrichment: \blacklozenge , P/0; \square , P/4; \blacktriangle , P/2; \circ , P/1.

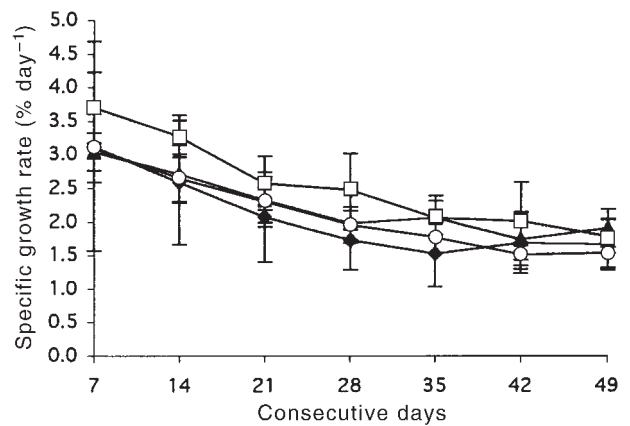


Fig. 4. Average specific growth rate in primary axis length of *Bostrychia moritziana* 3571 (female) after phosphorus enrichment: \blacklozenge , P/0; \square , P/4; \blacktriangle , P/2; \circ , P/1.

ficantly dependent on the concentration of phosphate available ($P < 0.05$, $P < 0.001$) (Fig. 6). The *B. moritziana* 3571 showed that all four concentrations of phosphate had the same effect on growth rate ($P > 0.05$) (Fig. 8).

Weekly observations of algal color and morphology indicated some of the impacts of nutrient-limitation. In the N-series of *B. moritziana* 2748, all plants in all

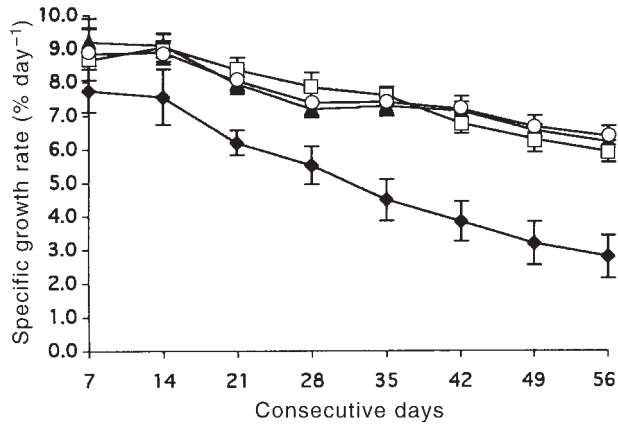


Fig. 5. Average specific growth rate in surface area of *Bostrychia moritziana* 2748 (female) after nitrogen enrichment: \blacklozenge , N/0; \square , N/4; \blacktriangle , N/2; \circ , N/1.

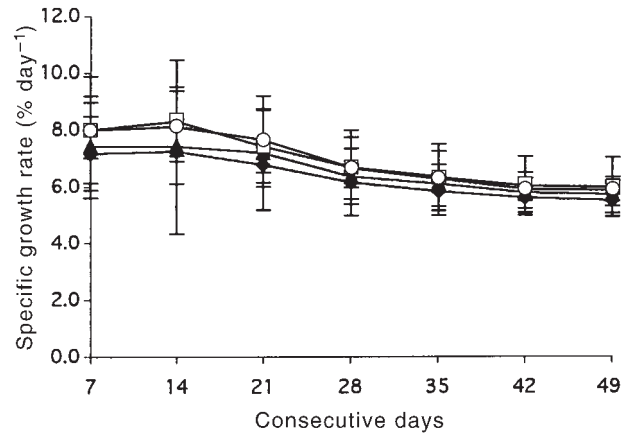


Fig. 8. Average specific growth rate in surface area of *Bostrychia moritziana* 3571 (female) after phosphorus enrichment: \blacklozenge , P/0; \square , P/4; \blacktriangle , P/2; \circ , P/1.

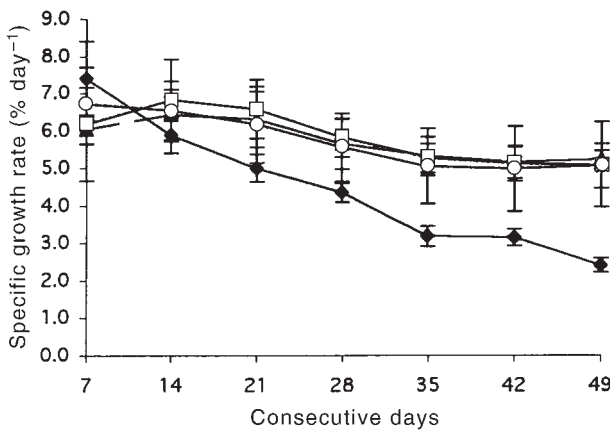


Fig. 6. Average specific growth rate in surface area of *Bostrychia moritziana* 3571 (female) after nitrogen enrichment: \blacklozenge , N/0; \square , N/4; \blacktriangle , N/2; \circ , N/1.

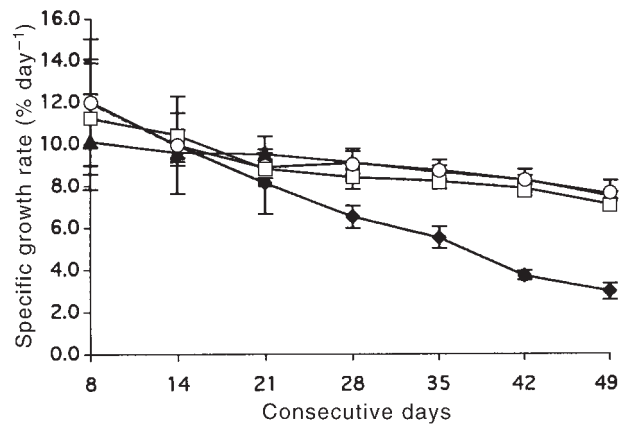


Fig. 9. Average specific growth rate in surface area of *Caloglossa lepieurii* 3543 (female) after nitrogen enrichment: \blacklozenge , N/0; \square , N/4; \blacktriangle , N/2; \circ , N/1.

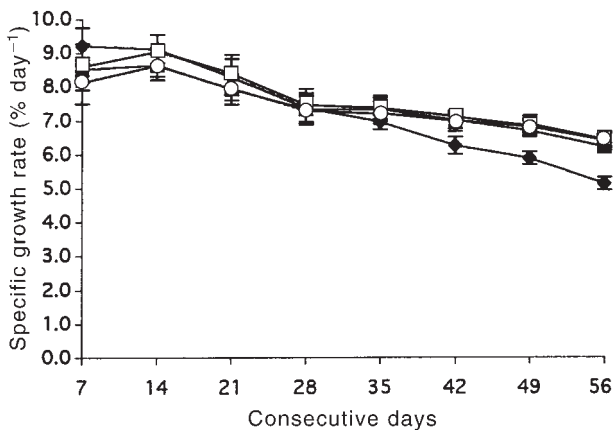


Fig. 7. Average specific growth rate in surface area of *Bostrychia moritziana* 2748 (female) after phosphorus enrichment: \blacklozenge , P/0; \square , P/4; \blacktriangle , P/2; \circ , P/1.

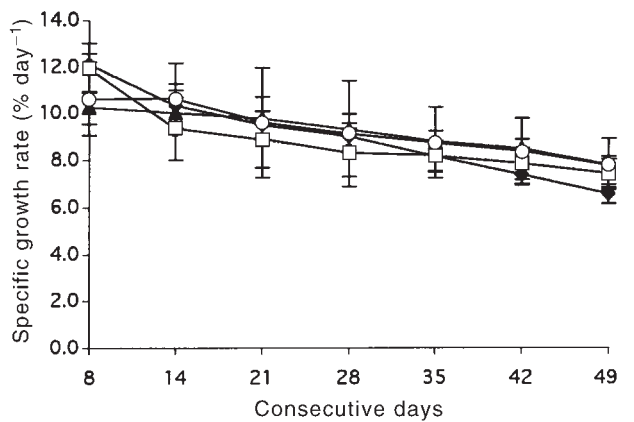


Fig. 10. Average specific growth rate in surface area of *Caloglossa lepieurii* 3543 (female) after phosphorus enrichment: \blacklozenge , P/0; \square , P/4; \blacktriangle , P/2; \circ , P/1.

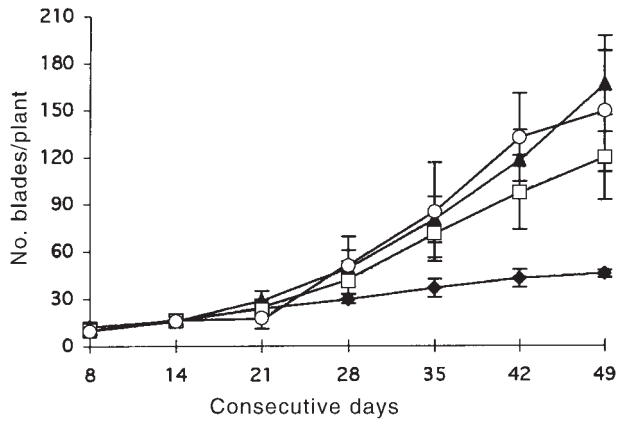


Fig. 11. The number of blades per plant of *Caloglossa leprieurii* 3543 (female) after nitrogen enrichment: ◆, N/0; □, N/4; ▲, N/2; ○, N/1.

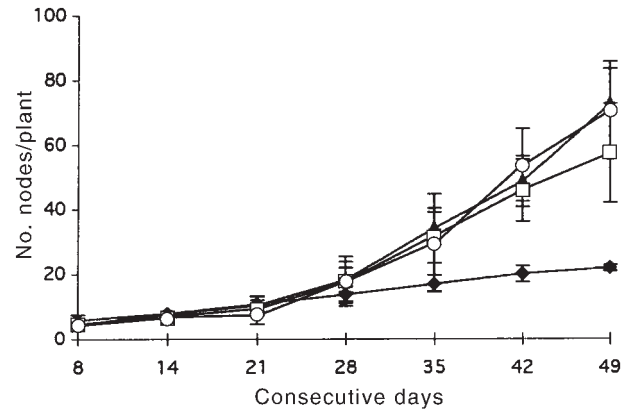


Fig. 13. The number of nodes per plant of *Caloglossa leprieurii* 3543 (female) after nitrogen enrichment: ◆, N/0; □, N/4; ▲, N/2; ○, N/1.

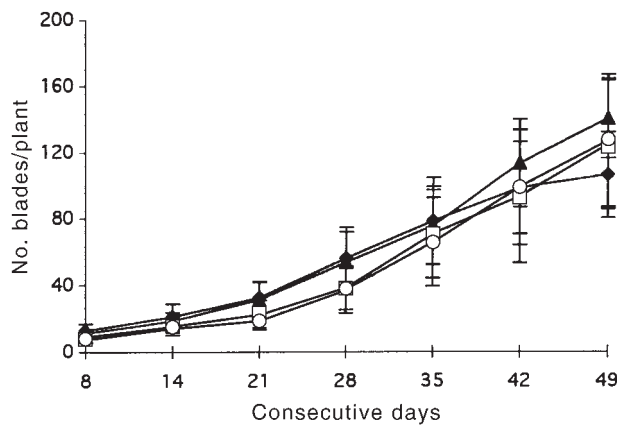


Fig. 12. The number of blades per plant of *Caloglossa leprieurii* 3543 (female) after phosphorus enrichment: ◆, P/0; □, P/4; ▲, P/2; ○, P/1.

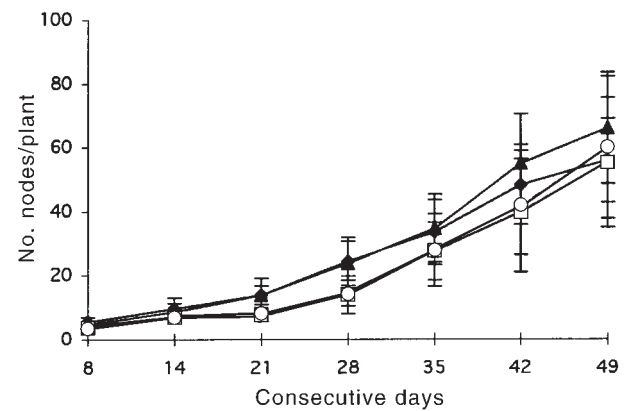


Fig. 14. The number of nodes per plant of *Caloglossa leprieurii* 3543 (female) after phosphorus enrichment: ◆, P/0; □, P/4; ▲, P/2; ○, P/1.

conditions looked healthy at $t = 7$ days. The N/0 plants at $t = 14$ days began to bleach out although the apices remained fully pigmented and healthy. This bleaching process continued until the end of the experiment and followed a distinct trend from lighter shades of reddish-brown, to varying shades of green and then yellow, and finally nearly translucent. When the plants became very pale, the apices lost their healthy appearance and cell death occurred. The N/4 and N/2 plants followed these same general trends, but the changes were not as rapid as in N/0. The N/1 plants generally remained healthy throughout experiments with only some slight bleaching occasionally occurring at $t = 49$ days. The *B. moritziana* 3571 plants followed the trends found in *B. moritziana* 2748, but seemed to progress at a slower rate overall in the bleaching process.

The physical appearance of *Bostrychia* in the N series (Fig. 15) corresponded well with the dramatic decrease in nitrate concentrations available to the

algae in the medium over the course of the experiments (Table 4). The bleaching increased with decreasing initial concentrations of nitrate and may be due to the corresponding period of N limitation. Additionally, the N/0 plants were always notably smaller than the plants in the other three conditions and with far less extensive branching.

In the P-series, all algae maintained a healthy appearance at $t = 7$ days. For the P/0 plants, some light bleaching began at about $t = 14$ days to 21 days. These plants became progressively paler over time with some having especially pale tips. The P/4 plants followed this same trend except for a slower loss of pigmentation. The P/2 plants appeared healthy until $t = 28$ days to 35 days. Later they became lighter and at $t = 49$ days they were greenish-brown. The P/1 plants remained healthy throughout the experiment. Again, the *B. moritziana* 3571 seemed more resistant to this overall bleaching process than the 2748 isolate.

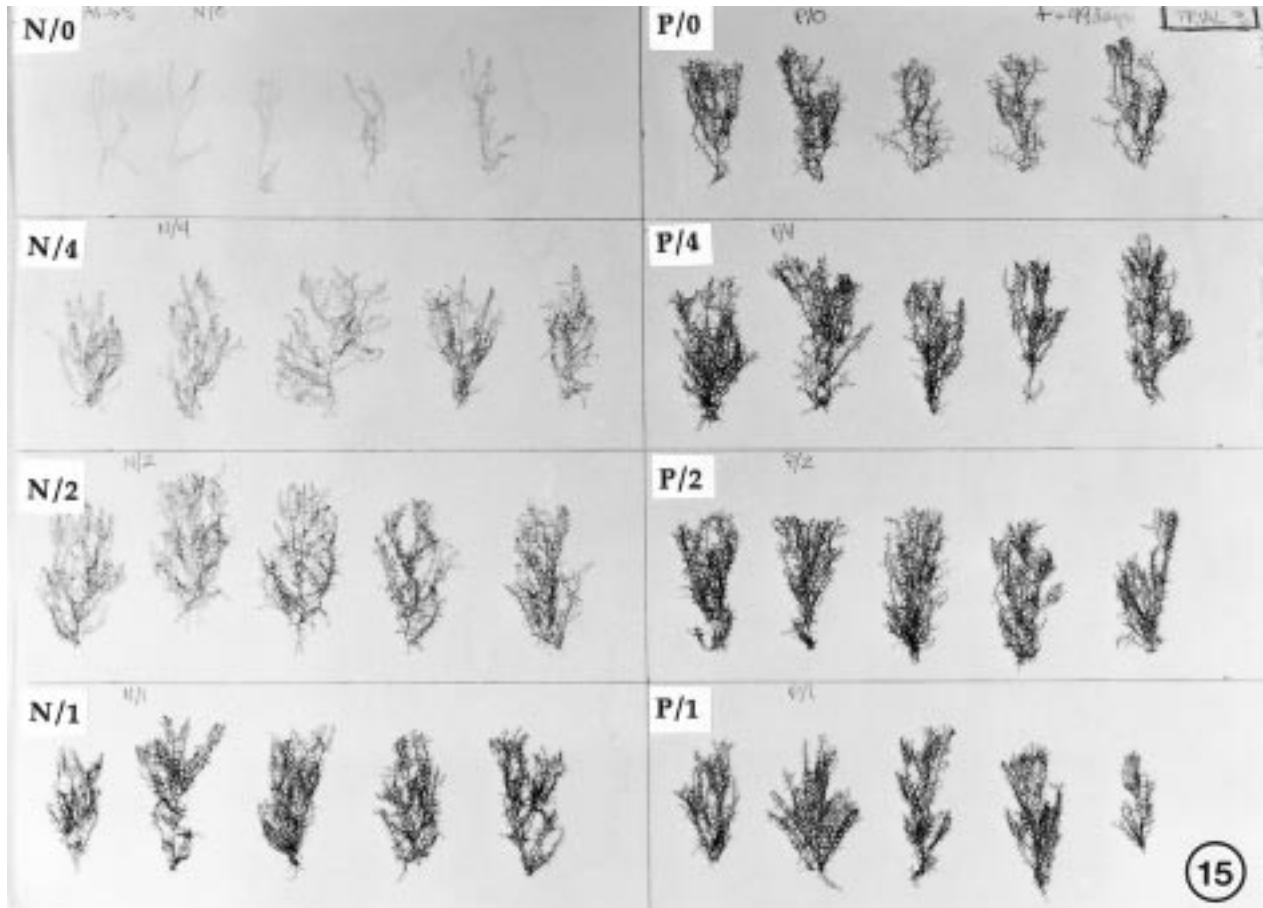


Fig. 15. *Bostrychia moritziana* grown in various phosphate and nitrate levels as indicated in Tables 2 and 3. Five replicates per condition were used and these fully grown specimens were pressed on an herbarium sheet. The N/0 specimens are very pale due to phycobilin pigment loss. The scale bar represents 1 cm.

Initial and final concentrations of phosphate in the seawater (Table 5) corresponded well with the physical appearance of the algae (Fig. 15). The P/0 plants were the most bleached and also the smallest in overall size (length and surface area) after the start of the experiment (with seawater levels declining from $83.8 \mu\text{g-P.L}^{-1}$ at $t = 7$ days to $7.7 \mu\text{g P L}^{-1}$ at $t = 49$ days). The remaining algae (P/4, P/2, and P/1) developed in size and shape as would be expected of a healthy plant; the only exceptions being the differences in rates of pigmentation loss as described above and the wide variations in the amount of phosphate each absorbed from the seawater during the course of the experiment (Table 5).

In the N series using *C. leprairiei* 3543, results showed strong evidence ($P < 0.001$) that specific growth rate in surface area was highly dependent on the nitrate concentration available in the ambient seawater (Fig. 9). Specific growth rates for all four conditions at $t = 8$ days were approximately $11\text{--}13\% \text{ day}^{-1}$, but N/0 declined to approximately $3\% \text{ day}^{-1}$ whereas the others declined to only approximately $7\text{--}8\% \text{ day}^{-1}$ by $t = 49$ days. In the

P series, surface area specific growth rate was significantly dependent ($P < 0.05$) on the P levels (Fig. 10). Specific growth rates for all four conditions again began at approximately $11\text{--}13\% \text{ day}^{-1}$ at $t = 8$ days, but all declined to $7\text{--}8\% \text{ day}^{-1}$ at $t = 49$ days.

In *C. leprairiei* 3543, the number of blades and nodes produced per plant increased with increasing N enrichment (Fig. 11) and statistical analysis provides very strong support ($P < 0.001$) of this. All four P levels (Figs 12 and 14) had the same effect on the number of nodes and the number of blades per plant ($P > 0.05$).

As with *Bostrychia*, the physical appearance of the *C. leprairiei* indicated the progression of nutrient-limitation (Fig. 16). The N/0 plants remained healthy until $t = 21$ days when they became pale yellow. Although the plants became lighter with time, the newly developing blades remained healthy until $t = 28$ days. At the end of the experiment ($t = 49$ days), the plants appeared nearly translucent. In addition, the N/0 plants were smallest and had the least amount of new blade formation of the four N-conditions (Fig. 11). The N/4 and N/2 plants

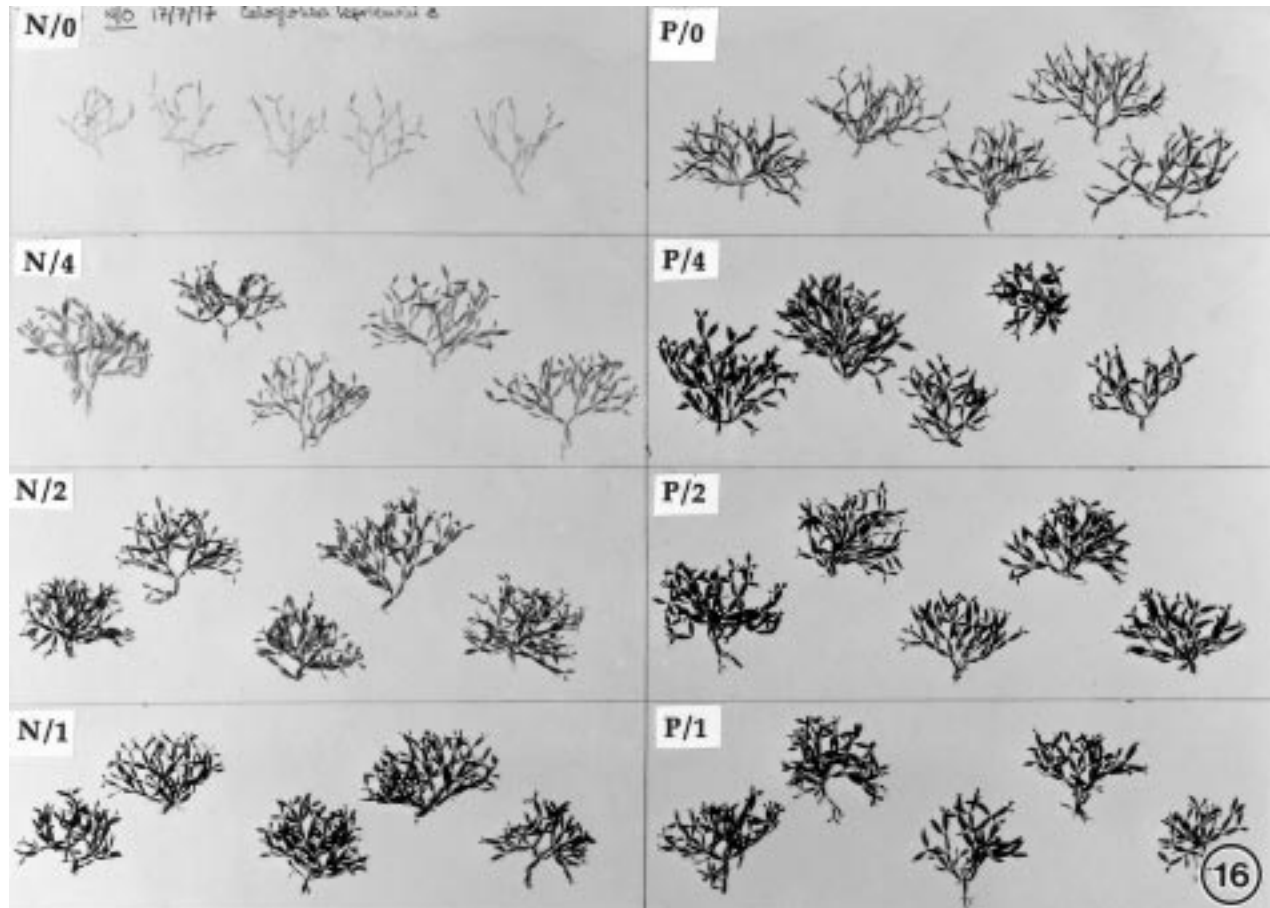


Fig. 16. *Caloglossa leprieurii* grown in various phosphate and nitrate levels as indicated in Tables 2 and 3. Five replicates per condition were used and these fully grown specimens were pressed on an herbarium sheet. The N/0 specimens are very pale due to phycobilin pigment loss. The scale bar represents 1 cm.

followed a similar, but less severe pattern than the N/0 plants (i.e. bleaching did not commence until $t = 35$ days or $t = 42$ days, appearing light yellow to light reddish-brown at $t = 49$ days). The N/1 plants were healthy throughout the experiment. Plants in these three N levels exhibited development in size and blade proliferation which was typical of a healthy plant. These results all corresponded well with the amount of nitrate available in the ambient seawater (Table 4). Despite having had different initial N-levels in the seawater (N/0 had $64.5 \mu\text{g N L}^{-1}$, N/4 had $1514 \mu\text{g N L}^{-1}$, and N/2 had $2900 \mu\text{g N L}^{-1}$), all resulted in N-levels of less than $10 \mu\text{g N L}^{-1}$ at $t = 49$ days.

In the P-series, the P/0 plants were healthy until $t = 21$ days when they became slightly lighter in color. At $t = 42$ days, the plants were yellowish and remained so until $t = 49$ days. Newly developed blades were healthy until $t = 49$ days when they had become a lighter reddish-brown. Initial and final concentrations of phosphate in the seawater (Table 5) corresponded with these physical characteristics (Fig. 16). The P/0 plants were the most bleached in color and the smallest in

size with a decline in seawater P levels from $83.8 \mu\text{g P L}^{-1}$ at $t = 8$ days to $9.5 \mu\text{g P L}^{-1}$ at $t = 49$ days. The P/4 plants appeared healthy until $t = 28$ days. They then became slightly lighter and remained pale until the end of the experiment. The P/2 and P/1 plants were healthy throughout the experiments. All three conditions had relatively high amounts of P remaining in the seawater at $t = 49$ days (P/4 had $34.4 \mu\text{g P L}^{-1}$, P/2 had $318.7 \mu\text{g P L}^{-1}$, and P/1 had $463.9 \mu\text{g P L}^{-1}$).

The two additional trials using a synthetic seawater medium (marine culture medium) in place of the natural seawater were not successful (data not shown). With *B. moritziana* 2748, the growth rates in primary axis length were nearly all negative (i.e. the plants were perceived by the computer to be reducing in size) at $t = 7$ days and remained like this until the experiment was terminated at $t = 21$ days. Surface area specific growth rates reached negative numbers only on the last day. The amount of bleaching was already extensive at $t = 7$ days. By $t = 21$ days, the apices still generally had some traces of color left in them although the rest of the plant was completely colorless. With *C. leprieurii*

3352, the trial lasted only 7 days because the bleaching had become so severe that the number of blades and nodes could not be discerned. At $t = 7$ days, the growth rates in surface area were all in the negative. Many plants had healthy looking apical regions on the newest blades although the rest of the plant was completely colorless. The situation only worsened with time.

DISCUSSION

The present study of *Bostrychia moritziana* and *Caloglossa leprieurii* indicated that the specific growth rates of these mangrove algae decrease with limiting concentrations of nitrate and phosphate.

For *Bostrychia* and *Caloglossa*, specific growth rates were highly dependent on the initial nitrate concentrations. Similar results were found in a variety of algal species. N-limitation was found to decrease growth in *Cladophora* sp. (Gordon *et al.* 1981), *Gracilaria foliifera* (Forsskål) Børgesen and *Ulva* sp. (Rosenberg and Ramus 1982), *Gracilaria tikvahiae* McLachlan (Lapointe and Duke 1984), *Ulva fenestrata* Postels et Ruprecht, *Codium fragile* (Suringar) Hariot, and *Pelvetiopsis limitata* Gardner (Wheeler and Bjornsater 1992). The time it took to show reduced growth rates due to N limitation in these algae varied from 7 days to 4–5 weeks. In our experiments, the algae started to show the effects of N limitation at 2 weeks.

The slower bleaching of *B. moritziana* 3571 versus 2748 may be attributed to the difference in the final N levels of the medium in 2748 versus 3571 (N/1 series, Table 4). In 2748, N dropped from 6080 to 223 $\mu\text{g N L}^{-1}$ (94% decrease) and in 3571, N decreased from 6080 to 3084 $\mu\text{g N L}^{-1}$ (51% decrease). However, in both series the specific growth rate was close to 1.0–1.5% day^{-1} at $t = 49$ days versus approximately 3.5% day^{-1} at the start. By contrast, the final P levels in 2748 and 3571 were quite close (402 and 468 $\mu\text{g P L}^{-1}$, respectively, Table 5) with a similar, corresponding decline in growth rate (from approximately 3.5% day^{-1} at $t = 7$ days to approximately 1.5% day^{-1} at $t = 49$ days).

In determining specific growth rates, the results of this study indicated a discrepancy between data from measuring primary axis length versus surface area. Growth in primary axis length appeared to be less accurate in assessing the total biomass of the alga and resulted in significantly lower growth values. For use in modeling systems, this would result in widely different data. For example, in the N/O trial with *B. moritziana* 2748 (Fig. 1), the primary axis growth rate began at about 3.5% day^{-1} at $t = 7$ days and declined to about 1.0–1.5% day^{-1} at $t = 49$ days, whereas the surface area growth rate (Fig. 5) was about 8.0% day^{-1} at $t = 7$ days and declined to about 3.0% day^{-1} at $t = 49$ days.

Color and morphology of the algae seem to be a good indication of N-limitation in both *B. moritziana* and

C. leprieurii. The increasing severity of bleaching over time in inverse proportion to the initial N level probably demonstrated the exhaustion of nitrate in the seawater with a concomitant decrease in pigment content. Other authors (DeBoer 1981; Bird *et al.* 1982; Fredriksen and Rueness 1989; Chopin *et al.* 1995) have found strong correlations between N levels and the algal appearance/pigment reduction. Gordon *et al.* (1981) found that in N-free media, *Cladophora* underwent distinct chlorosis after 5 weeks. N-limited *Gracilaria* plants became bleached over time from a dark red to a red-orange (Lewis and Hanisak 1996). In *Gracilaria*, amino acids and proteins are a major nitrogen storage pool with phycoerythrin pigments becoming an important nitrogen reserve under initially nitrogen-limited conditions (Bird *et al.* 1982). Due to this relationship between N limitation and the reduction in phycobilin pigment content, the well-known bleaching of red algae may be due to nitrogen deficiency as well as the effects of the sun (Lignell and Pedersen 1987; Fredriksen and Rueness 1989). The large temporal fluctuation in nutrient supply to the mangrove environment would give those algae which have nutrient-storage capabilities an advantage over algae without them.

In addition, the lack of nitrate reduced branching frequency in plants in N/O when compared with those in higher N levels. There is a strong correlation between meristem growth rate and the number of lateral branches (Fredriksen and Rueness 1989). The meristematic regions of kelps have the highest demand for N (Lobban and Harrison 1994). Chapman and Craigie (1977) found that the mature blade tissue in *Laminaria longicuris* showed dramatic effects of the seasonal variation in tissue N, while the new tissue forming in meristematic regions was utilizing the available N. Likewise, in *Bostrychia* and *Caloglossa*, the branch apices remained pigmented during stages of N-limitation while older branches lost pigmentation. Unfortunately, tissue analyses for N and P were not possible in this study due to the small sample size (≤ 20 mg dry weight).

The effect of initial phosphate concentration on growth rate was varied. In the four different P concentrations for *B. moritziana*, there was no significant difference in the growth rate of primary axis length. The surface area growth rate of *B. moritziana* 2748 was dependent on the P level, while that of *B. moritziana* 3571 was not affected by the P level. In *C. leprieurii*, the surface area growth rate was dependent on the initial phosphate concentration of the seawater. In most algal literature, there is a significant correlation between growth rate and P levels (e.g. Chopin *et al.* 1990). A decrease in growth rate was noted after 4 weeks at P/O in *Cladophora* (Gordon *et al.* 1981), *Gracilaria* (Lewis and Hanisak 1996) and *Gelidium robustum* (Gardner) Hollenberg *et al.* (Sousa-Pinto *et al.* 1996). *Gracilaria* began to fragment at 6 weeks and went from red to orange to yellow in P/O. After

8 weeks, the *Gelidium* was described as 'doing poorly'.

Of the four P levels used in our studies, those algae in P/O were the smallest in size and the most bleached throughout the experiment. The remaining plants in P-limited conditions underwent few physical changes until about the third week. Results indicated that phosphate is not very limiting to *Bostrychia moritziana* and *Caloglossa leprieurii*. Phosphate levels would have to be severely reduced in the seawater for longer than 56 days (the longest time period monitored in this study) in order to stop growth. An occurrence such as this is highly unlikely in the field. However, *B. moritziana* and *C. leprieurii* are capable of assimilating P from seawater and the results of this study warrant further investigation into the possibility that these algae can create internal reserves that will supply sufficient P in all but the most severe environmental conditions. This situation is known in other marine algae such as *Cladophora* which is reportedly capable of storing large amounts of P during non-limiting conditions (Gordon *et al.* 1981). Overall, these results indicate that phosphate is in far less demand as a nutrient than nitrate in these mangrove algae, but may still limit their growth rate under more extreme circumstances.

The preconditioning period can also have effects whereby the previous rate of nutrient supply may influence the rate of nutrient uptake (Caperon and Meyer 1972; Fujita 1985). Regardless of where the *Bostrychia moritziana* and *Caloglossa leprieurii* were originally collected, they were maintained in laboratory-culture using seawater collected from the pier of the Williamstown Anglers Club on Port Phillip Bay. Water analysis of the unenriched seawater indicated a molar ratio of 2:1 N:P (excluding ammonia in the determination of N levels) ($75.7 \mu\text{g N L}^{-1}$ and $85.1 \mu\text{g P L}^{-1}$). The N levels fell within the 'normal' range of $1\text{--}500 \mu\text{g N L}^{-1}$ (Sverdrup *et al.* 1942; Riley and Chester 1971). The P levels, however, were extremely elevated and above the $1\text{--}35 \mu\text{g P L}^{-1}$ 'standard' range (Sverdrup *et al.* 1942; Riley and Chester 1971; Hernandez *et al.* 1997). It is possible that the levels of P in the seawater were too high to have allowed the initial 7-day 'starvation' period in this study to be effective. If so, this may account for why there seemed to be little effect of the various P-enrichments on growth. It is also likely that this over-abundance of P in Port Phillip Bay is why Lewis (1973) found the concentration of tissue-P in the algae of Port Phillip Bay to be so stable while the levels of N were subject to changes in relation with seasons, and location in relation to shore position and point sources of pollution. Based on the water nutrient levels of this experiment, it is also possible that the high levels of P already present in the bay water were further enriched by the single nutrient pulse and that this may have resulted in P-saturation of the algae. This would explain why, for example, there was a great difference in final water P-concentrations between the P/O and

P/4 versus P/2 and P/1 conditions. The fact that the dried tissue samples were too small for nutrient analysis means that we cannot be sure if this or some other hypothesis explains the results.

The synthetic seawater medium MCM has proven useful in growing other red, brown and green algae (Woelkerling *et al.* 1983; Hunt and Mandoli 1996), but was not satisfactory as a replacement for the natural seawater medium with *B. moritziana* and *C. leprieurii*. For whatever reason, in the MCM they bleached extensively within the first week and had negative growth rates.

In conclusion, the results presented here show that *B. moritziana* and *C. leprieurii* are prone to N limitation, with P limitation possible but less likely. The digital imaging technique developed here to measure the growth of these algae proved to be a considerable improvement for accurate representations of the overall changes in growth over the more conventional methods available. Direct application of this procedure can be used for any number of subjects and purposes in a wide variety of fields.

Although we took all practical measures to control variables, there is of course still the possibility that factors other than those described may have contributed to the results obtained. Having only looked at the effects of a single nutrient pulse following a 7-day 'starvation' period, there remains a tremendous variety of experiments yet to do in furthering our understanding of the nutrient requirements of mangrove algae.

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