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MORPHOLOGICAL VARIATION OF
CHAMPIA PARVULA (C. AGARDH) HARVEY IN SYNTHETIC MEDIUM
WITH SPECIAL ATTENTION TO THE INFLUENCE OF BROMIDE

BY

MARY BETH ABEL

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

BIOLOGICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

1999

ABSTRACT

This study investigated the effectiveness of a self-paced learning program. Results demonstrated that the self-paced program was significantly more effective than the traditional classroom program.

MASTER OF SCIENCE THESIS

OF

MARY BETH ABEL

The grade of this student was a highly satisfactory one. The student demonstrated a high level of achievement in all areas of the program. The student's work was of a high quality and was well presented. The student's work was of a high quality and was well presented. The student's work was of a high quality and was well presented.

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1999

ABSTRACT

While under investigation as a toxicity-testing organism, female gametophytes of the small marine red alga, *Champia parvula* (C. Agardh) Harvey exhibited a loss of diaphragm formation in indeterminately growing branch tips. Further study revealed that this change of morphology occurred when bromide was omitted from nutrient enriched, synthetic seawater (Thursby and Harlin 1984).

The goals of this project were to verify and further document the loss of diaphragm formation in *C. parvula* under bromide minus and other culture conditions and to observe developmental events associated with the loss of diaphragm formation. The working hypothesis was that diaphragm formation is dependent on bromide. To test this hypothesis, diaphragm formation in *C. parvula* branch tips was morphometricly measured by monitoring first segment length (FSL), the region from the apex to the first observable diaphragm. Overall growth of *C. parvula* branch tips was monitored by measuring tip length (TL), the distance from the apex to the base of the tip. FSL was selected as an appropriate measure of diaphragm formation by comparing FSL to the increase in segment number in branch tips over time. Tips in synthetic media lacking bromide showed an increase in FSL, but only a small increase in segment number indicating that formation of new diaphragms and, therefore, new segments had stopped. In contrast, branch tips in control treatments showed an increase in segment number but little change in FSLs over time indicating continuous, periodic formation of new diaphragms. The sensitivity of diaphragm formation to bromide availability was observed in both tetrasporophytes and female gametophytes in two versions of synthetic media. However, the two versions of synthetic media with bromide (used as controls)

proved unsuccessful in maintaining diaphragm formation indefinitely. Toward the end of experiments, FSL was inconsistent among branch tips cultured in synthetic media, and by two weeks, branch tips from synthetic media were more similar in length to tips from the bromide free media than to tips from NSW. This finding undermined the hypothesis that loss of diaphragm formation depends on the availability of bromide.

Lack of bromide may induce stress in *C. parvula* and loss of diaphragm formation may be a generalized stress response. This possibility is supported in that *C. parvula* showed a reduced reproductive capability in synthetic media as compared to natural seawater, and that altering nitrogen sources led to loss of diaphragm formation. Features of *C. parvula* that were not significantly affected in synthetic media with and without bromide were growth (as measured by TL), branch formation, and the development of hyphal filaments and gland cells.

The effort to test whether bromide is required for diaphragm formation in *C. parvula* was not conclusive. However, the investigation did support previous research performed with *C. parvula* -- developmental work by Bigelow (1887) and culturing studies by Thursby and Harlin (1984). A staining technique was tried in the investigation that, if pursued, may provide the histological information that is needed to determine the role of bromide in diaphragm formation in *C. parvula*. Suggestions for pursuing this research in the future are provided.

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Before I began this writing stage of my degree, I thought about what I would say in my acknowledgments. It is here that I can document my good experience of finding people who helped me by sharing their equipment, advice, and time. The following friends and acquaintances have been helpful to me and I would like to sincerely express my gratitude for their kindness. They are as follows: Dr. Martha Apple; Dr. Eve Arnold; Mike Bono; Dr. John Babson; Dr. Bob Bullock; Shannon Donovan; Colleen Driscoll; Missy Englehard; Dr. Bill Euler; Dr. Roger Goos; Dr. Paul Hargraves; Dr. Jim Heltshe; Dr. David Heskett; Sarah Higbie; Dr. Linda Hufnagel; Dr. Tom Hull; Paul Johnson; Nina Kajiji; Dr. Keith Killingbeck; Dr. Richard Koske; Brian Kozell; Sangkug Lee; Christopher Nerone; Dr. Lucy Maranda; Peter Morgan; Mike Peck; Paul Pennington; Jennifer Prentice; Dr. Eric Roberts; Colleen Rose; Dr. Dennis Rhoades; Dr. Eileen Sadasiv; Stephanie Schollaert; Steve Schott; Brian Setlik; and Dr. Elijah Swift. Scientists outside of the URI community who kindly offered me assistance are: Dr. Annette Coleman; Dr. Michael Gretz; Dr. Gary Saunders; Dr. Valerie Vreeland; and my high school biology teacher, Dr. Glenda George.

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INTRODUCTION

Champia parvula (C. Agardh) Harvey is a small, marine red alga with a cosmopolitan, subtidal distribution (Taylor 1957, Lewis 1973, Abbott and Hollenberg 1976). The morphology of this alga may be described as a branched, segmented tube. A single layer of cortical cells form the walls of the tube whereas single layers of cells, called diaphragms or septa, intersect the tube at regular intervals (Bigelow 1887, Davis 1892).

When *C. parvula* is switched from nutrient-enriched natural seawater to nutrient enriched synthetic seawater lacking bromide, diaphragm formation at branch tips eventually ceases (Thursby and Harlin 1984). The result is an apparent lengthening of the first segment (the region from the apex to the first observable diaphragm) as the alga continues apical growth. Over time, aseptate branch tips become flattened and misshapen (Thursby and Harlin 1984).

Although much literature exists on the presence of halogenated secondary metabolites in algae, the role of halides (*i.e.*, chloride, bromide, and iodide) in the metabolic processes is not well understood (*i.e.*, Fenical 1975, Butler and Walker 1993). Proposed metabolic roles for halides include the maintenance of osmotic potential (Fenical 1975), incorporation into secondary metabolites that inhibit bacterial growth, epiphytism or herbivory (Butler and Walker 1993); and synthesis of haloperoxidases or halogenated phenolics that may be involved in cross-linking of polymeric substances (Johnson *et al.* 1995, Vreeland *et al.* 1998). A handful of studies have documented morphological variation in macroalgae relative to the availability or concentration of halides (Fries 1966, Wolk 1968, Hsiao 1969, Fries 1975, McLachlan 1977). With the

exception of a study of stalk formation in a diatom (Johnson *et al.* 1995), no studies have investigated the relationship of the development of a morphological feature in an alga relative to the availability of a halide.

Bigelow (1887) studied the morphology of *C. parvula* collected from the wild and built upon the understanding of the structure of this alga by comparing his findings to the work of previous investigators. Through careful examination of sectioned and whole mount *C. parvula*, Bigelow observed that diaphragms are formed about 30 μm from the apex and proposed that diaphragm cells arise from hyphal filament cells which themselves form from the divisions of apical cells. As the apical cells repeatedly divide first anticlinally (perpendicular to the branch axis) and then periclinally (parallel to the branch axis), the cells toward the exterior of the branch become cortical tissue whereas cells toward the inside become the first hyphal cells. These initial cells are elongated compared to cortical cells and compose uniseriate filaments that span the length of branch tips. Early on in their development, near the apex, hyphal filament cells, located at intervals around the interior of the branch apex, either bud a small round cell (the gland cell) or another “outgrowth”, a cell that will give rise to a diaphragm. These cells, coming from each hyphal filament cell, meet in the center of the thallus forming the diaphragm (Bigelow 1887).

Building on the work of Thursby and Harlin (1984) and Bigelow (1887), the goals in this investigation were to verify and document the loss of diaphragm formation in *C. parvula* under bromide-free and other culture conditions and to observe developmental events associated with the loss of diaphragm formation. The working hypothesis was that diaphragm formation is dependent on bromide. This phenomenon is easily studied in *C.*

parvula. The alga exhibits quick, indeterminate growth permitting its continuous clonal propagation in laboratory culture (Steele *et al.* 1986, Thursby and Steele 1986).

MATERIALS AND METHODS

General information about *Champia parvula* (C. Agardh) Harvey (Rhodophyta, Rhodymeniales)

Source tissue used in this investigation included a non-spore producing tetrasporophyte, originally obtained from the Environmental Research Laboratory, Environmental Protection Agency (Narragansett, Rhode Island) and a female gametophyte obtained from Science Applications International Corporation (Narragansett, Rhode Island) in 1996. The tetrasporophyte isolate stopped producing spores in culture in 1995. Both of these isolates originated from *C. parvula* collected in 1979 from Ninigret Pond, a coastal salt pond, in Rhode Island (Steele *et al.* 1986). The tetrasporophyte never resumed tetraspore production during these experiments, and the possibility exists that this isolate underwent a spontaneous mutation. It has been reported that *C. parvula* exhibits spontaneous mutation of genes for pigmentation and morphology (Steele and Thursby 1980, Thursby and Steele 1986). For example, on fertilized, female yellow mutants (alleles designated as *yell-1*, *yell-2*, and *yell-3*), carposporophytes failed to develop normally and to produce viable carpospores (Steele *et al.* 1986).

The triphasic life cycle of *C. parvula* involves isomorphic and free-living female and male gametophytes and a tetrasporophyte. The third phase, the carposporophyte, is reduced and essentially parasitic on the female gametophyte and develops subsequent to

fertilization (Bold and Wynne 1985, Steele *et al.* 1986, Thursby and Steele 1986).

Trichogynes (reproductive hairs) on female gametophytes collect spermatia cast by male gametophytes; carposporophyte development follows and carpospores, produced within the carposporophyte, give rise to tetrasporophyte individuals. The production of tetraspores from tetrasporophyte plants continues the life cycle; male and female gametophytes arise from these spores, released as tetrads.

Stock culture of *C. parvula*

Stock cultures of *C. parvula* were maintained in nutrient enriched natural seawater (NSW) and cultured as previously described with few modifications (Thursby and Steele 1986, Apple 1994, Apple and Harlin 1995) (see Appendix A for nutrient enrichment recipe). NSW, filtered with 10 μm and 1 μm cartridges used in succession, was collected at the Environmental Research Laboratory, Environmental Protection Agency (Narragansett, Rhode Island) in 10 liter and 20 liter Nalgene carboys on an incoming high tide. Collected NSW was then autoclaved at 18 p.s.i., 121 C for 30 - 45 min. (20 liter carboys were autoclaved 3 at a time; 10 liter carboys were autoclaved at 4 - 6 at a time). The autoclaving time sufficiently eliminated algal contaminants throughout the investigation. NSW was typically 30 ppt, however, when salinity was higher, deionized water was used to adjust the salinity to 30 ppt after autoclaving and prior to culturing. Autoclaved NSW was kept in cool, dark storage until use. Contaminants that appeared in the autoclaved sea water included rod shaped, gram negative bacteria (personal observation), fungal hyphae (possibly *Aspergillus* sp., personal communication with Dr. Roger Goos, Department of Biological Sciences, URI), and a choanoflagellid

and a kinetoplastid, both nonphotosynthetic protists (identified by Dr. P. Hargraves, Graduate School of Oceanography, University of Rhode Island, personal communication). Cutting back tissue and changing stock cultures on a weekly basis inhibited the presence of these contaminants. It is worth noting that bacteria are considered to be beneficial, and perhaps even required, for normal macroalgal growth and development in culture (Provasoli and Pinter 1980, Dworetzky 1983).

Glassware was washed with RBS-35 (Pierce Chemical Co., Rockford, IL) and then rinsed thoroughly in hot tap water followed by distilled water. Residue from the detergent was removed by soaking glassware in a 1:5 solution of concentrated HCl: distilled water. After acid treatment, glassware was rinsed thoroughly in distilled water. Prior to use, all glassware was autoclaved for 16 min. at 121° C, 18 p.s.i. (Apple and Harlin 1995).

Cultures were kept at 22° C ± 2° C in Percival Growth Chambers on a 16 h:8 h light:dark cycle. Lighting from above was 14 - 17 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from cool-white fluorescent lights. Measurements of lighting were made with a Quantum/Radiometer/Photometer, Model L1-189 (Li-Cor, Lincoln, NB).

Experimental culturing conditions

Experimental cultures were treated in the same manner as stock cultures (Thursby and Steele 1986, Apple 1994, Apple and Harlin 1995) with a few exceptions. Culture medium was aerated continuously and changed every 5 or 7 days. During experiments, flasks were randomly repositioned in the culture chambers on a daily basis. Lighting from above and below using cool-white fluorescent lights was either 30 - 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$

or $14 - 22 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$. The latter light level was achieved with shade cloth.

The recipes for nutrient enrichment solutions and for synthetic seawater media are provided in Appendix A. Two nutrient enriched synthetic seawater media were used during the investigation: Sm based on McLachlan (McLachlan 1973; personal communication with Alexander O. Frost) and St used by Thursby and Harlin (Thursby and Harlin 1984). All media and nutrient enrichment solutions were made using reagent grade chemicals. Table 1 lists shorthand notation for the media and treatments used in the investigation. Table 1A (Appendix B) organizes the details of all experiments for comparison.

Cultures of *C. parvula* branch tips for all experiments were maintained in five-hundred milliliter flasks containing 500 ml of aerated treatment or control medium. The number of branch tips (2 –3 mm in length) added to each culture flask at the beginning of an experiment depended upon the duration of the experiment. Typically, experiments lasted for 7, 10 or 14 days with sampling on Days 1, 2, 3, 5, 7, 10 and 14, depending on the experiment. On sampling days, six tips were randomly collected from each flask with a sterile glass pipette or a stainless steel scoopula cleaned with 70% ethanol. Five of the collected tips were measured; an extra tip was always collected in case one tip was damaged during collection.

Prior to all experiments, morphometric measurements and descriptive information about the appearance of tissue was recorded for a sample of branch tips. The means \pm 2S.E. for Day 0 values per experiment are listed according to corresponding figure number in Table 1A (Appendix B).

Table 1: Notation for media used in the investigation

Refer to the Appendix A for complete recipes and descriptions of nutrient enrichment solutions and synthetic media. For Sm and St, "X" represents a trace metal in a solution of trace metals or the solution itself, referred to as TM. The components of TM are aluminum (Al), bromide (Br), iodide (I), lithium (Li), rubidium (Rb), and strontium (Sr). The salinities range from 28-30 ppt.

Notation	Medium	Bromide conc.	Source or Type
NSW	Natural sea water, nutrient enriched	814 uM	Environmental Research Laboratory, EPA, RI
Sm	Synthetic sea water, nutrient enriched	290 uM	based on McLachlan (1973)
St	Synthetic sea water, nutrient enriched	739 uM	Thursby & Harlin (1984)
Sm-X and St-X	Sm or St minus a single trace metal or a trace metal solution	see above	see above
StFC	same as St; St with ferric chloride, normal addition	" "	" "
StFS	St with ferric chloride replaced by ferric sulfate	" "	" "
SmNO ₂	Sm with nitrate replaced by nitrite	" "	" "

A number of morphological features were monitored in an early experiment. The features measured for branch tips of the tetrasporophyte isolate included tip length, the number of segments per tip, the individual lengths and widths of all segments per tip, and the perimeters of cells composing each segment per tip (Figure 1). In experiments following this initial work, first segment length (FSL), the lengthwise distance from the apex of a branch tip to the first diaphragm, and tip length (TL), the distance from the apex of a branch tip to its base, were primarily measured. In one experiment, tetrasporophyte and female branch tips were cultured for 20 days with the media changed on Day 10. Treatments included NSW, Sm, and Sm-Br (Table 1). At the end of the culturing period, the tissue per treatment was blotted and weighed.

Specific Experiments

a. *Manipulation of synthetic media (Sm): trace metals*

Components of the trace metal solution (TM) were individually eliminated and the effects on tip and first segment lengths for tissue from the *C. parvula* tetrasporophyte isolate were monitored. The elements in TM included aluminum (Al), bromide (Br), iodide (I), lithium (Li), strontium (Sr), and rubidium (Rb) (Table 1A, Appendix B). Additional treatments included Sm-TM, and Sm and NSW, as controls (Table 1).

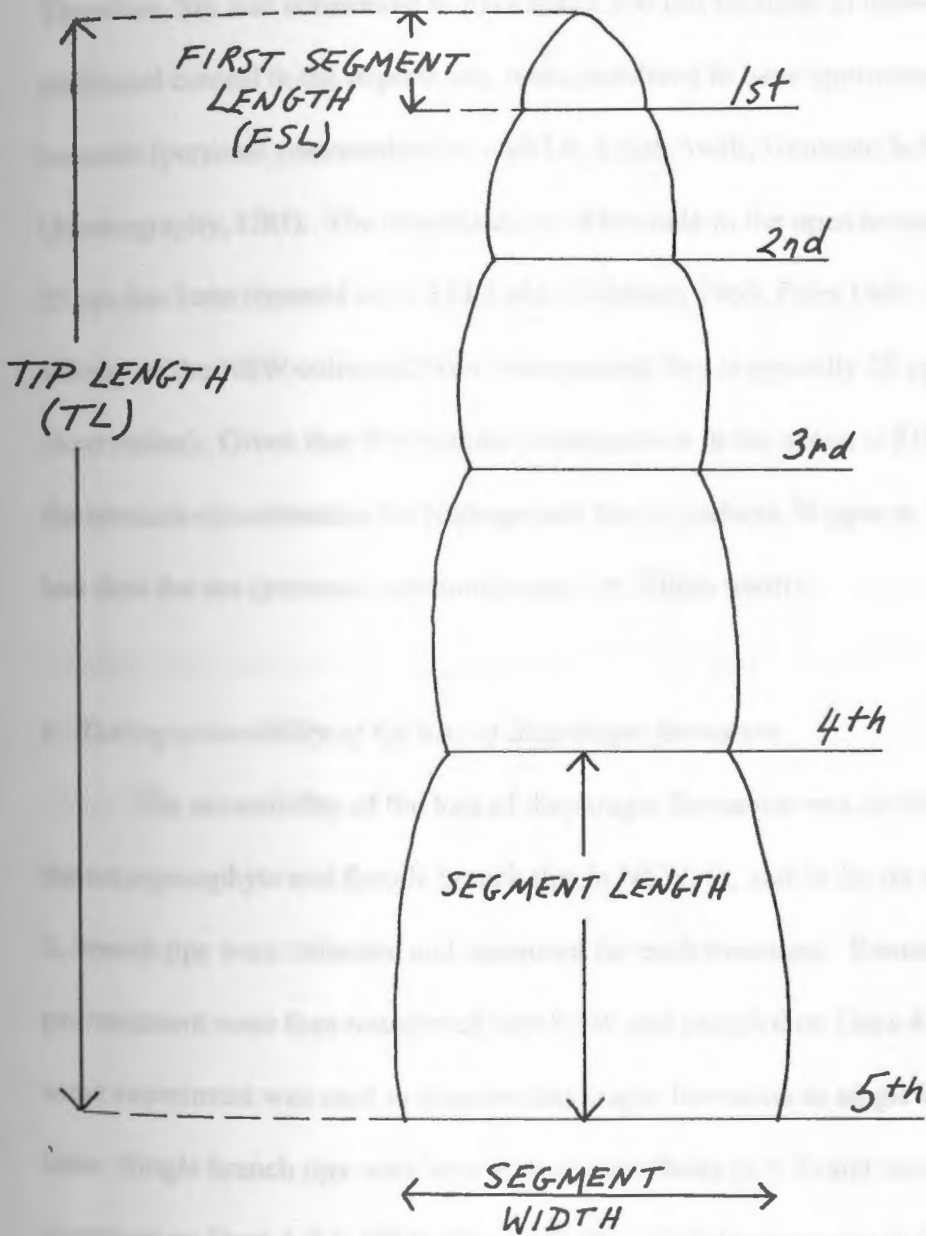
b. *Manipulation of synthetic media (Sm): bromide concentration*

To determine the required level of bromide concentration to maintain diaphragm formation in the tetrasporophyte isolate, a range of concentrations was tested using Sm. Added as NaBr, these were: 0 uM, 25 uM, 50 uM, 100 uM, 150 uM, and 200 uM.

Figure 1

Schematic diagram of *C. parvula*

The first segment, as newly formed tissue, serves as a reference point for numbering older segments from the apex to the base of the branch tip. Five segments are represented. Abbreviations that are used in the text for morphometric measurements are: FSL = first segment length and TL = tip length. Additional features measured in branch tips were segment length, segment width and cell perimeter for each segment. Scale: 10 cm = 1 mm. The branch tip in this diagram represents a 1.47 mm long tip with a first segment length at approximately 0.13 mm.



$$10\text{cm} = 1\text{mm}$$

The concentration of bromide in complete Sm was approximately 290 μM . Adding NaCl and KCl to Sm potentially contributed an additional 10 μM bromide as contamination. Therefore, Sm was considered to have about 300 μM bromide in experiments. NSW, an additional control in the experiment, was considered to have approximately 700 μM bromide (personal communication with Dr. Elijah Swift, Graduate School of Oceanography, URI). The concentration of bromide in the open ocean with a salinity of 35 ppt has been reported to be 814.3 μM (Goldberg 1963, Fries 1966, Fries 1975). The salinity of the NSW collected from Narragansett Bay is typically 30 ppt (personal observation). Given that the bromide concentration in the ocean is 814.3 μM (65 ppm), the bromide concentration for Narragansett Bay is perhaps 56 ppm or 700 μM , about 14% less than the sea (personal communication, Dr. Elijah Swift).

c. Testing reversibility of the loss of diaphragm formation

The reversibility of the loss of diaphragm formation was investigated by treating the tetrasporophyte and female branch tips in NSW, St, and St-Br for three days. On Day 3, branch tips were collected and measured for each treatment. Remaining branch tips per treatment were then transferred into NSW and sampled on Days 4, 5, 6, and 8. This same experiment was used to observe diaphragm formation in single branch tips over time. Single branch tips were kept in treatment flasks ($n = 3$) and monitored both during treatment on Days 1-3 in NSW, St, and St-Br and during recovery in NSW on Days 4, 5, 6 and 8.

d. *Investigating additional causes of loss of diaphragm formation*

During the investigation, additional culturing experiments were performed to improve upon techniques for culture of *C. parvula* in synthetic media. The goal of this effort was to reduce the variability seen in the data obtained for tissue treated in Sm. This variability was most noticeable on the latter days of experiments and usually larger than that seen for tissue cultured in NSW. The following features of experimental culturing were tested. Lighting was adjusted from 30 - 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to 14 - 22 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using shade cloth. Media was changed more frequently on Day 5 compared to Day 7. Finally, the synthetic media used initially, Sm, was replaced with St, the version of synthetic medium used by Thursby and Harlin (1984) (Appendix A). One experiment using St involved trying out a suggested alternative source of iron. *C. parvula* tetrasporophytes and female gametophytes were cultured in St with the usual source of iron, ferric chloride, and in St with a suggested alternative, ferrous sulfate (Dr. Glen Thursby, Environmental Research Laboratory, Environmental Protection Agency, Narragansett, Rhode Island, personal communication). The concentration of iron in both St treatments was 350 μM . The influence of culture in synthetic media on reproduction and branch formation was also examined.

Finally, an experiment was performed to see how diaphragm formation would be affected by replacing nitrate with nitrite as a source for nitrogen. This form of nitrogen was thought to induce loss of diaphragm formation in *C. parvula* (Dr. Glen Thursby, personal communication). Female tissue was used in the experiment. Nitrate and nitrite were added to St as sodium salts in concentrations of 1.5 mM and 1.8 mM, respectively.

Image analysis

Microscopic image analysis of whole mounts and sections of *C. parvula* branch tips was performed with either the 4x objective of an Olympus inverted research microscope, model IMT, or the 10x objective on an Olympus compound microscope, model Vanox. A Hitachi CCTV camera was used with both microscopes. Microscopic images were measured using a digitizing tablet and digitized using a IBM PCAT equipped with Microcomp image analysis software. Because branch tips were usually too long for the field of view through the microscope and on the monitor, a ruler was used to measure tip length in millimeters. First segment lengths that were too long to measure in one field of view were measured in parts.

In order to have a standard for comparison to cultured *C. parvula*, tetrasporophytes and female gametophytes were collected from the drift at Scarborough Beach (ScarB) and Pier #5 (Pier5), Narragansett, Rhode Island, in August 1997, and at Sheep's Point Cove (ShpPt), Newport, Rhode Island in July 1998. First segment length was measured for ten branch tips on each of six plants using image analysis. Additionally, branching patterns and the appearance of plants were documented. First segment length on female and tetrasporophyte branch tips averaged 0.105 ± 0.04 mm (n = 60) and ranged from 0.047 mm to 0.283 mm.

Statistical analysis

Values for each set of five tips were pooled with values from replicate flasks within a single experiment or from replicate runs of an experiment. Additionally, the tetrasporophyte and female isolates, cultured separately during an experiment, were

treated as replicates. Data was pooled once interaction effects were determined to be minimal or not significant by plotting the data and performing two-factor ANOVA using tissue type or experiment as one factor compared to either sampling day or treatment. Following this initial analysis for tissue type and replicate experiments, sampling day and treatment were used as factors in two-factor ANOVA for all experiments. In most cases, data was log (base e) transformed prior to ANOVA to both normalize and confer equal variance among treatments. However, in many cases, the data did not pass tests for normality and equal variance even after transformation, but treatment effects were often highly significant at $p \leq 0.01$. ANOVA is described as “robust” meaning it is resistant to Type 1 errors and yields accurate results for data sets with equal sample sizes (as was the case in these experiments) that are not normally distributed and that lack equal variance among means for treatments (Underwood 1981). The Student-Newman-Keuls test was used for multiple comparisons when the data was found to show significant differences ($p = 0.05$). Statistics were performed using SigmaStat statistical software (Jandel Scientific Corp., San Rafael, CA).

Histology

During experiments, tissue was collected on certain sample days and fixed in FAA (formalin-alcohol-acetic acid, see Appendix A) for 24 - 48 hours. This coagulative fixative prevented the delicate *C. parvula* tissue from collapsing during processing. Due to the fact that FAA does not preserve cytoplasm, cell outlines were easily distinguished in thick sections. Following fixation, tissue was dehydrated using the following, finely graded ethanol series: 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%,

95%, and two exchanges of 100%. The tissue was kept in labeled tubes to which the ethanol solutions were added. The tubes were continuously mixed on a rotary shaker at room temperature and each dehydration step lasted 30 minutes.

Tissue was infiltrated with JB-4 Plus embedding media (JB-4 embedding kit, Polysciences, Inc.) according to the manufacturer's instructions. Sectioning of blocks was performed on a Sorvall JB-4 microtome using glass knives. In some instances, pieces of the blocks were sawed off and reoriented to get cross-sections of tips; cyanoacrylate adhesive (*i.e.*, super glue) was used to reattach block pieces. Thick sections (2 - 5 μm) were transferred to filtered water droplets on glass slides, cleaned with 70% ethanol. Sections were dried down on the slides for at least a day using a warming plate.

The following stains were applied to sections, but were not used routinely due to the lack of contrast and lack of differential staining of classes of molecules: Saffranin/Fast Green; 1% Acid Fuchsin in distilled water; 0.1% Fast Green, pH 2; Alcian Blue/Alcian Yellow (Parker and Diboll 1966); 0.3% Alcian Blue w/v in 3% acetic acid, pH 2.5 (Apple 1994); Heath's Toluidine Blue, Methylene Blue and Neutral Red (Heath 1961). The strongest staining reaction, with the most contrast, occurred with 1% Toluidine Blue with 1% borax. Sections were stained by flooding slides for 1-3 min. on a warming plate. Slides were rinsed with distilled water and air-dried. A coverslip and mounting medium were not used to view sections; however, for photomicrography, sections were mounted in immersion oil that has the same refractive index as JB-4 Plus methacrylate.

In an attempt to trace cell lineage in branch tips, whole mounts of *C. parvula* were stained with DAPI fluorescent nuclear stain (Goff and Coleman 1986, Goff and Coleman 1987) and with aceto-iron-haematoxylin-chloral hydrate stain (Wittman 1965, Coomans and Hommersand 1990). Prior to staining with DAPI, tissue was fixed in 3:1 95 % ethanol: glacial acetic acid for a day or more, transferred to 70% chloral hydrate for 1 - 2 hours, and finally squashed on a glass slide (techniques modified from Goff and Coleman 1986, Goff and Coleman 1987). The squashed tissue was soaked in a small amount of 0.5 ug/ml DAPI in natural sea water plus GP2 enrichment solution (2 ml / 100 ml sea water) (see Appendix A) and microwaved for 10 sec. Afterward, the tissue was remounted in water and observed using brightfield and fluorescence microscopy. Tissue was also fixed in 3:1 95% ethanol: glacial acetic acid prior to staining with the aceto-iron-haematoxylin-chloral hydrate method. Following fixation, tissue was mounted in a glass slide and heated in a microwave oven for 10 sec. After excess fixative was removed, a drop of stain was added to the tissue on the slide. The slide was briefly heated over an ethanol burner until the edges of the stain turned mauve. The stain is made up of 5 ml of stock staining solution plus 2 g of chloral hydrate. This solution was filtered and then allowed to age for 24 hours prior to use. The stock staining solution consisted of 4 g of haematoxylin, 1 g iron alum ($\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$; a.k.a. ferric aluminum sulfate) (Wittman 1965).

RESULTS

Figures 2a and 2b compare the data across treatments for first segment and tip lengths for Day 3 and Day 14 of an experiment designed to repeat the work of Thursby and Harlin (1984). The effects of eliminating individual trace metals from nutrient enriched synthetic seawater were examined by making morphometric measurements of *C. parvula* tetrasporophyte branch tips over time. This experiment revealed that the length of the first segment, the distance from the apex to the first observable diaphragm, increased in branch tips cultured in synthetic seawater lacking a solution of trace metals (Sm-TM) and in synthetic seawater lacking only bromide (Sm-Br). By comparison, first segment lengths (FSLs) changed little over time in branch tips cultured in control treatments, nutrient enriched natural seawater and synthetic seawater, and in the other treatments (those with bromide). For example, the mean first segment length (FSL) for all tips on Day 0 of this experiment was 0.125 ± 0.06 (2S.E.) ($n = 40$), however, by Day 3, FSLs for tips cultured in Sm-TM and Sm-Br, nearly 1 mm, were significantly greater than the FSLs for tips cultured in the controls and in the other treatments (Figure 2a). In contrast, the overall tip lengths (TLs) for tips from the controls and all synthetic seawater treatments were not significantly different from each other (Figure 2a). By Day 14, FSLs for tips cultured in Sm-TM and Sm-Br were both over 4 mm and remained significantly greater than FSLs for the controls and other treatments (Figure 2b). However, on this sampling day, FSLs for Sm-TM and Sm-Br were also significantly different from each other on Day 3. Additionally, the FSLs for the other synthetic seawater treatments, including complete Sm, were all significantly greater than NSW. On Day 14, TL for

Figures 2a and 2b

Testing the effects of eliminating individual trace metals from Sm: Days 3 and 14

First segment and tip lengths for Day 3 (2a) and for Day 14 (2b) are compared across treatments. Bars represent means \pm 2S.E. (n = 10). (2a) First segment lengths (FSLs) for tips cultured in Sm-TM and Sm-Br were significantly different from other treatments but not from each other on Day 3 (2a). There were no significant differences among treatments for tip length on Day 3. (2b) Significant differences among treatments for Day 14 are treated separately for FSL and tip length (TL). Capital letters (TL) and lower case letters (FSL) identify treatments that are significantly different ($p < 0.0001$). Treatments with the same letter are not significantly different from each other.



Figure 2a

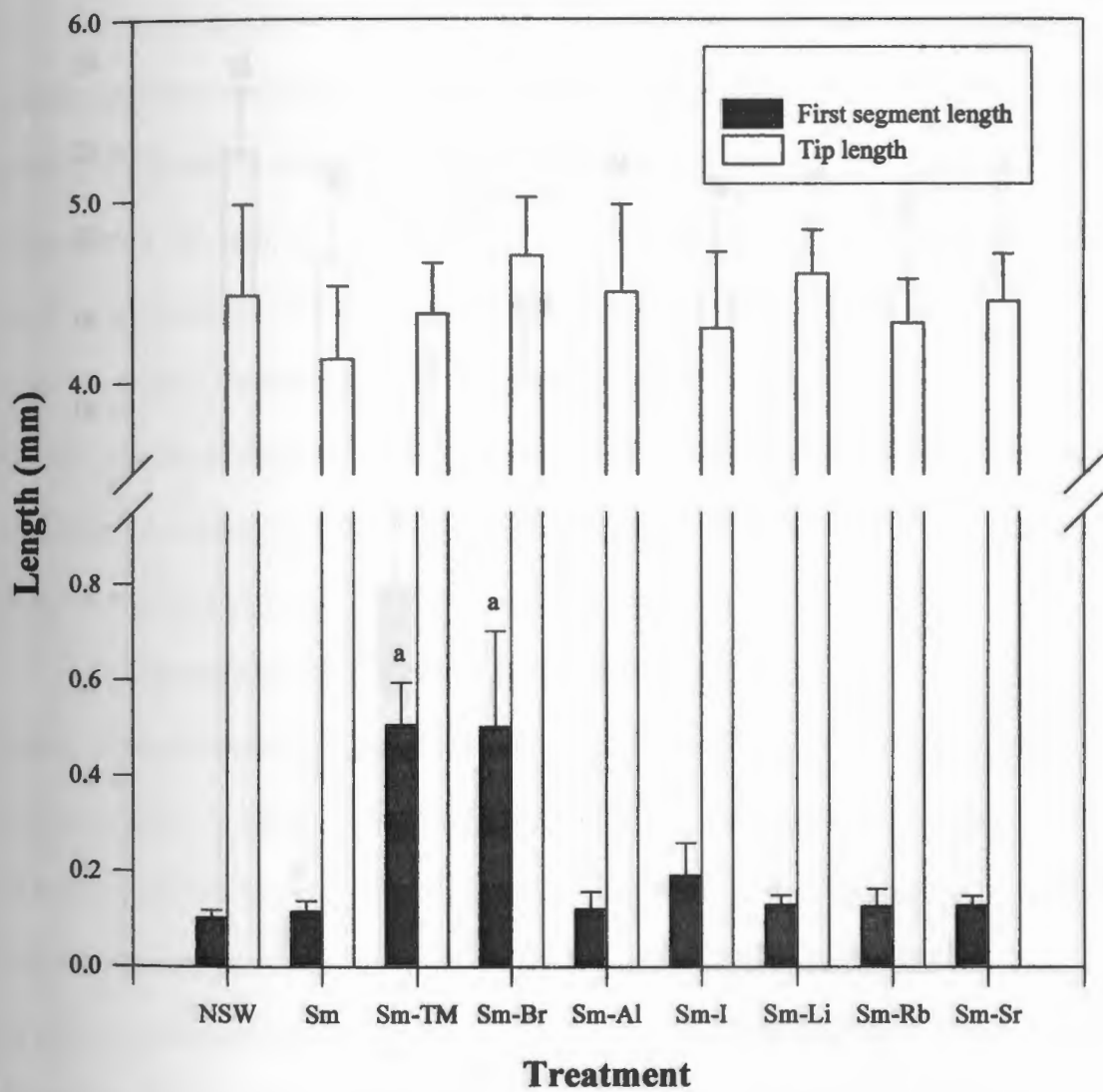
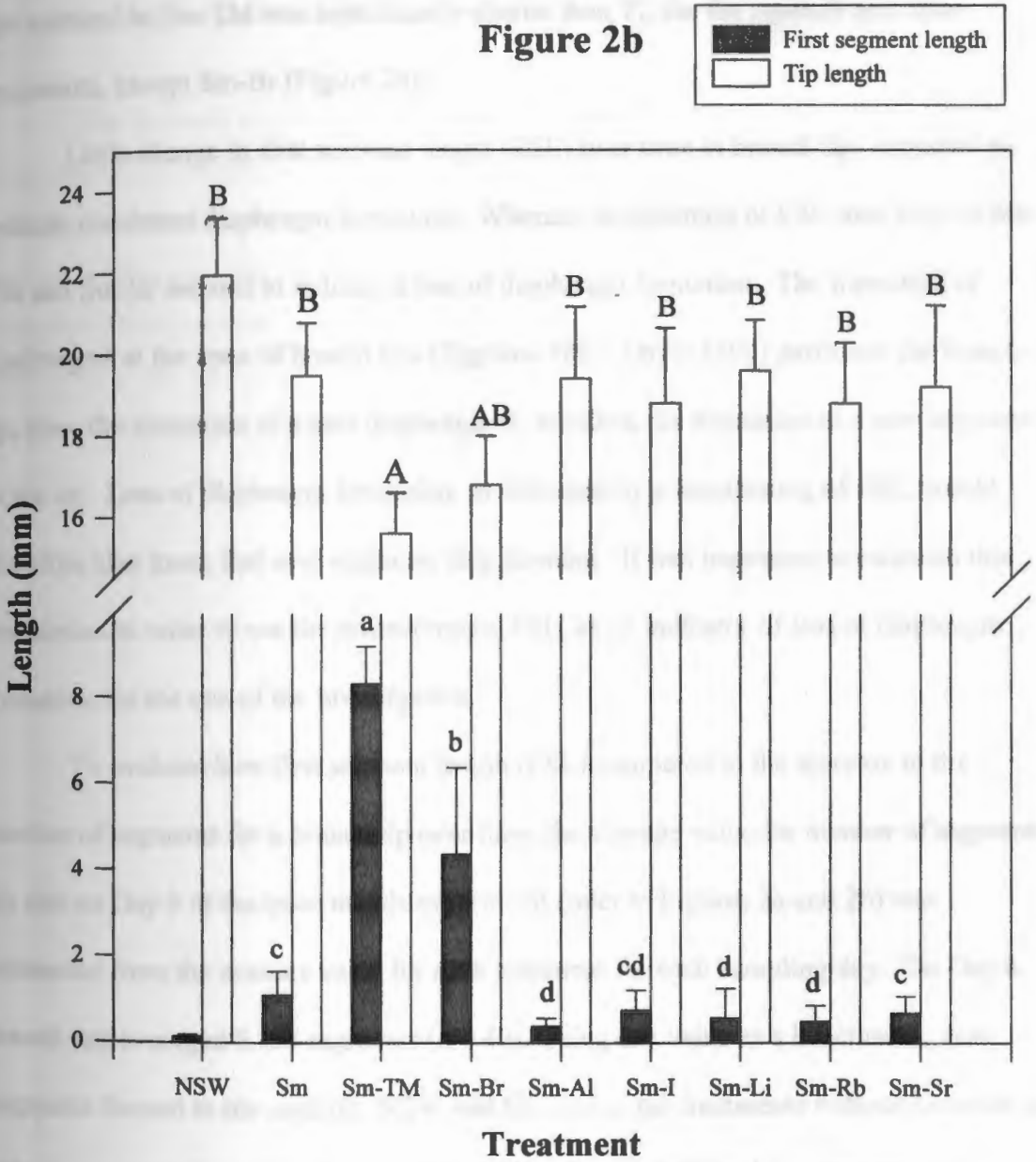


Figure 2b



tips cultured in Sm-TM was significantly shorter than TL for the controls and other treatments, except Sm-Br (Figure 2b).

Little change in first segment length (FSL) over time in branch tips appeared to indicate consistent diaphragm formation. Whereas, lengthening of FSL over time in Sm-TM and Sm-Br seemed to indicate a loss of diaphragm formation. The formation of diaphragms at the apex of branch tips (Bigelow 1887, Davis 1892) partitions the branch tip, thus, the formation of a new diaphragm is, in effect, the formation of a new segment in the tip. Loss of diaphragm formation, as indicated by a lengthening of FSL, would therefore also mean that new segments stop forming. It was important to establish this correlation in order to use the measurement, FSL, as an indicator of loss of diaphragm formation for the rest of the investigation.

To evaluate how first segment length (FSL) compared to the increase in the number of segments for a branch tip over time, the average value for number of segments for tips on Day 0 of the trace metals experiment (refer to Figures 2a and 2b) was subtracted from the average value for each treatment for each sampling day. On Day 0, branch tips averaged 5.155 segments ($n = 40$). Using this value as a benchmark, new segments formed in the controls, NSW and Sm, and in the treatments without bromide on subsequent sampling days. Additionally, FSL in the controls and these treatments remained fairly constant at less than 1 mm for the duration of the experiment. In contrast, the number of segments in tips from Sm-TM and Sm-Br remained fairly constant while FSL increased over time. Figure 3 exhibits data for Day 10 of the experiment (the data for the other sampling days is not shown). FSL and TL are compared to the number of

Figure 3

Comparing first segment and tip length to formation of new segments

First segment length and tip length for *C. parvula* tetrasporophyte branch tips are compared to the number of new segments formed by Day 10 of the experiment to test the effects of eliminating trace metals (refer to Figures 2a and 2b). Bars for the number of new segments (gray fill) represent values determined by subtracting the mean number of segments for the tips on Day 0 was 5.155 (n = 40) from the mean number of segments for Day 10 (n = 10). Bars for FSL (black fill) and TL (white fill) represent means \pm 2S.E. Significant differences mentioned in the text are based on $p < 0.001$.

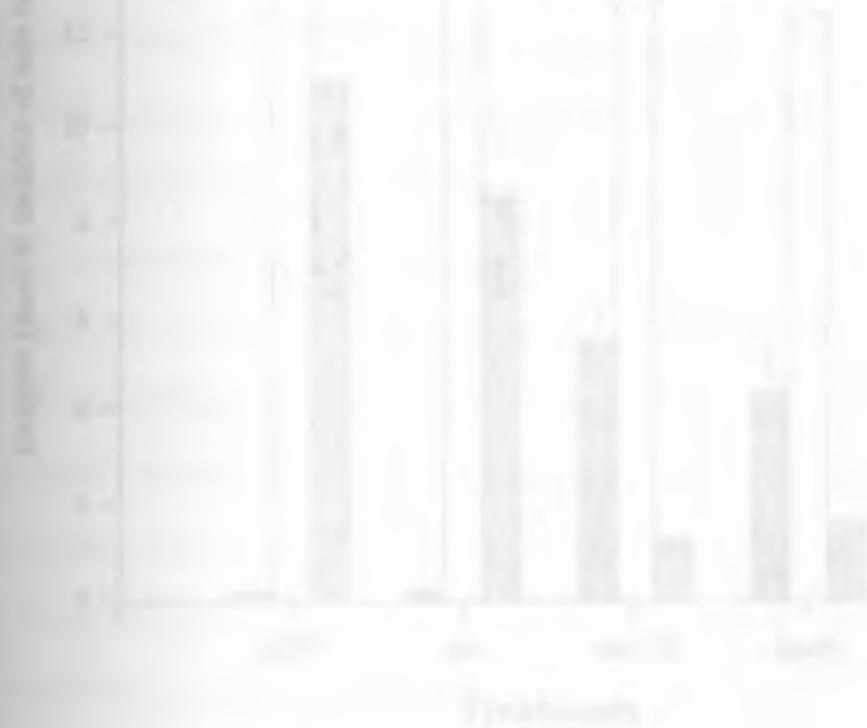
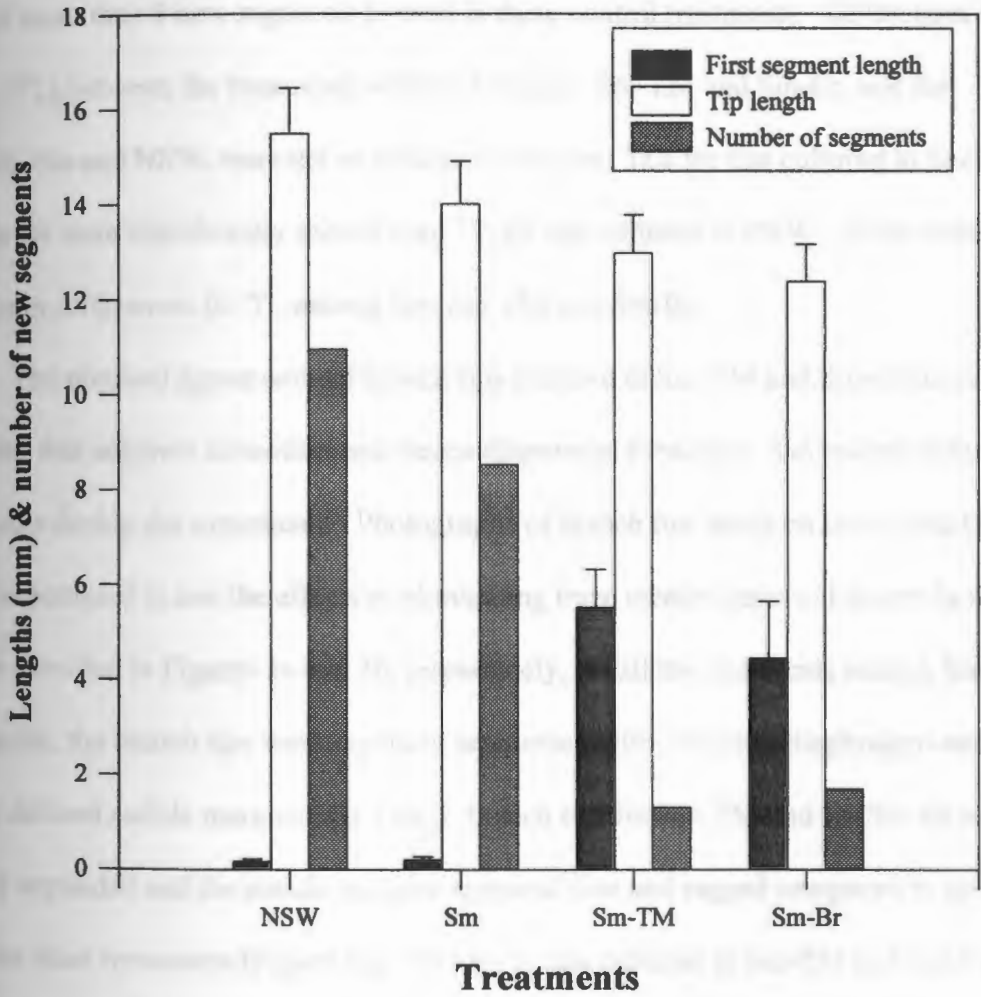


Figure 3



new segments since Day 0. FSLs in branch tips cultured in Sm-TM and Sm-Br are large having increased over time from approximately 0.17 mm on Day 1 to about 5 mm on Day 10. However, less than two new segments were produced in these branch tips by Day 10. In contrast, FSL did not change much over time in Sm and NSW (refer to Figures 2a and 2b) and more than 8 new segments formed in these control treatments. Differences in tip length (TL) between the treatments without bromide, Sm-TM and Sm-Br, and the controls, Sm and NSW, were not as distinct. However, TLs for tips cultured in Sm-TM and Sm-Br were significantly shorter than TL for tips cultured in NSW. There were no significant differences for TL among Sm, Sm-TM and Sm-Br.

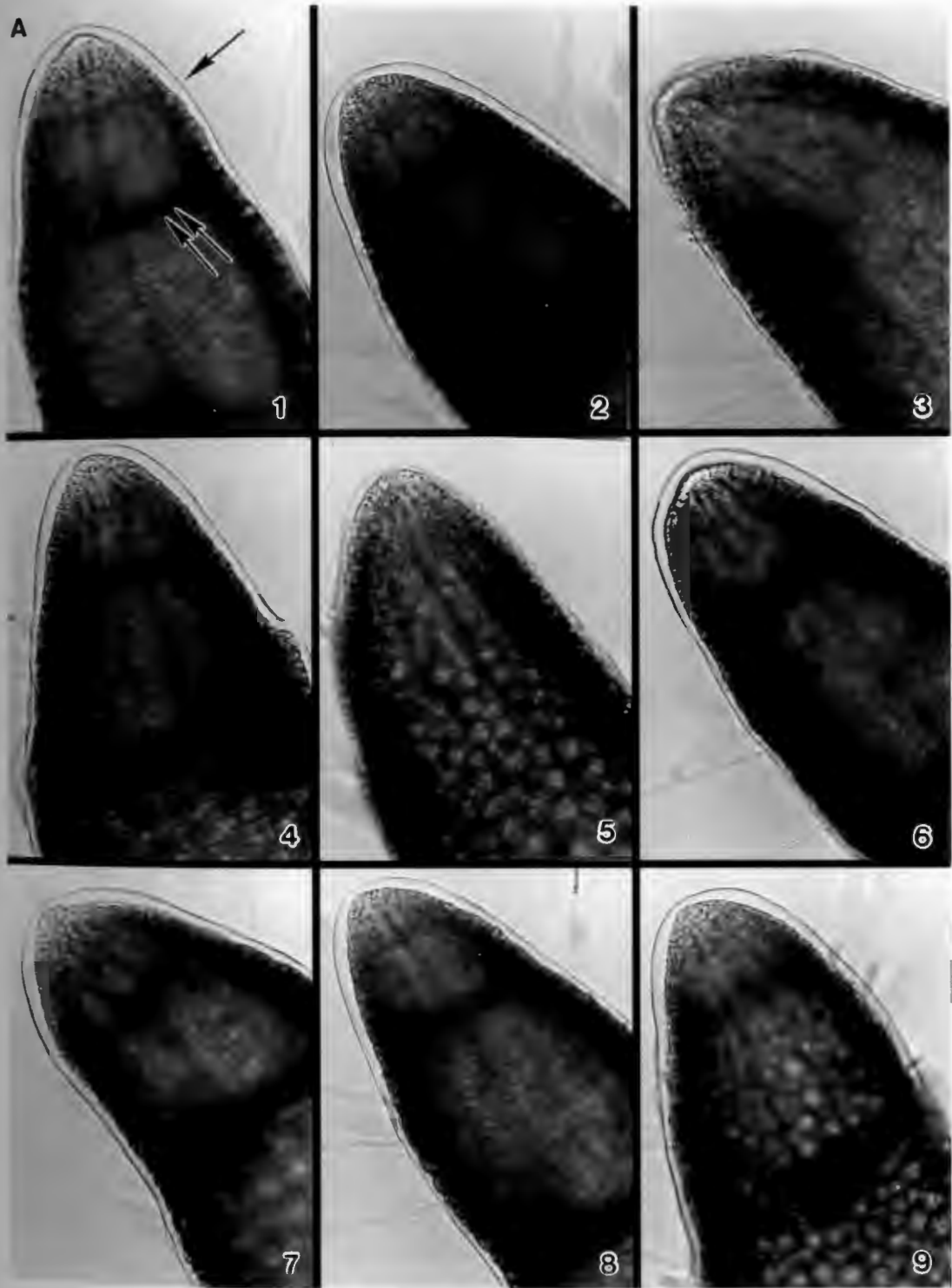
The physical appearance of branch tips cultured in Sm-TM and Sm-Br further indicated that segment formation, and hence diaphragm formation, had ceased in these treatments during the experiment. Photographs of branch tips taken on Day 5 and Day 8 of the experiment to test the effects of eliminating trace metals (refer to Figures 2a and 2b) are provided in Figures 4a and 4b, respectively. In all the treatments except, Sm-TM and Sm-Br, the branch tips were regularly segmented with complete diaphragms and clearly defined cuticle margins. By Day 5, branch tips for Sm-TM and for Sm-Br were slightly expanded and the cuticle margins appeared thin and ragged compared to tips from the other treatments (Figure 4a). By Day 8, tips cultured in Sm-TM and Sm-Br clearly lacked diaphragms at the apices which were expanded and misshapen (Figure 4b). Interestingly, new branches had formed in these tips, yet these too lacked diaphragms (personal observation).

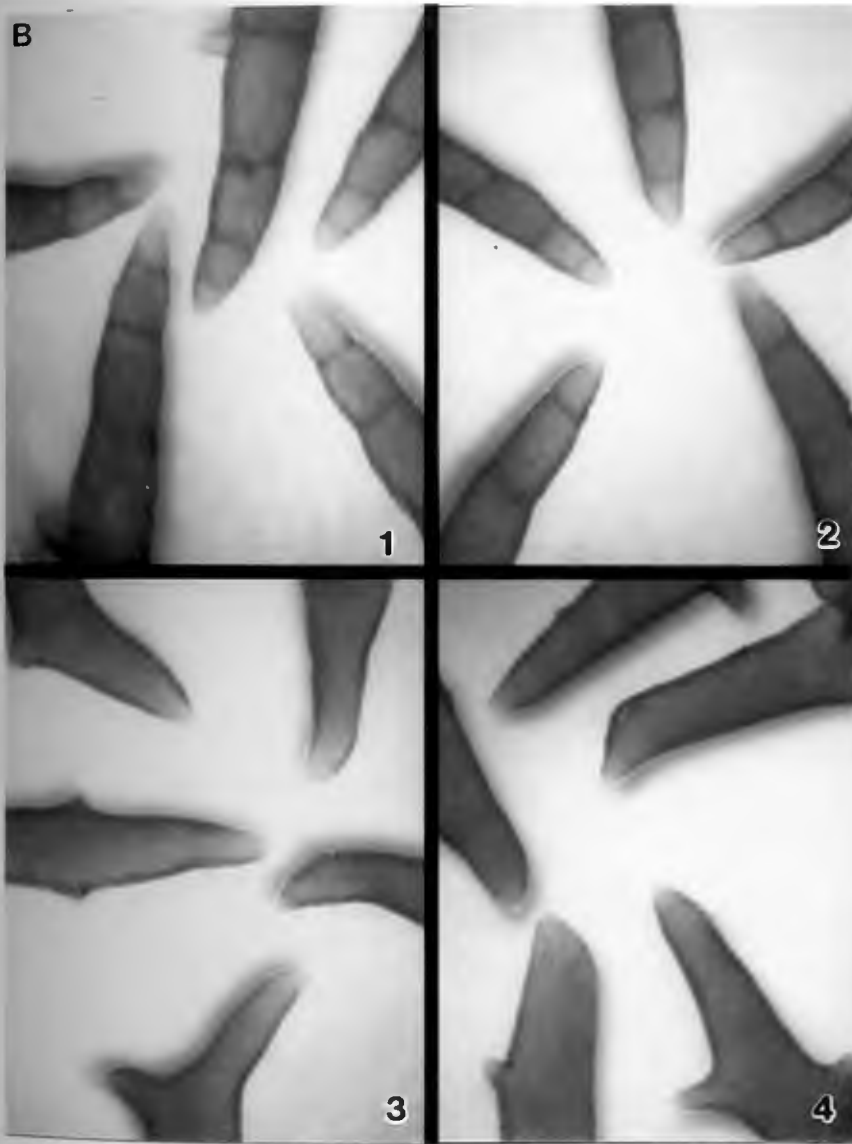
Diaphragm formation in *C. parvula* tetrasporophyte branch tips was supported throughout a ten day experiment with 50 μ M bromide in nutrient enriched synthetic

Figures 4a and 4b

The effects of eliminating individual trace metals from Sm: Days 5 and 8

(4a) Day 5 of the experiment. Treatments: 1. NSW; 2. Sm; 3. Sm-TM; 4. Sm-Al; 5. Sm-Br; 6. Sm-I; 7. Sm-Li; 8. Sm-Rb; 9. Sm-Sr. Photographs were taken at 10X using an Olympus BH-2 RFCA compound microscope. All tissue is magnified ~ 1200X. In each case, tips were brought into focus so that the outline of the cuticle margin was clear (arrow). Diaphragms, seen as darkened lines perpendicular to the branch axis (double arrows), are absent in the branch tips of the Sm-TM (4a-2) and Sm-Br (4a-5) tissue, and the cuticle margins in these treatments are relatively thin and discontinuous in comparison to the margins of branch tips from the other treatments. (4b) Day 8 of the experiment. Treatments: 1. NSW; 2. Sm; 3. Sm-TM; 4. Sm-Br. Photographs were taken at 2X (with 10X projector lens) using a Nikon SMZ-10 stereo microscope and a Nikon FX-35 camera back. Final magnification is about 75X. Branch tips are misshapen in Sm-TM (4b-3) and Sm-Br (4b-4) due to the loss of diaphragm formation.





Figures 5a and 5b

Testing the effects of manipulating bromide concentration in Sm

The effect of bromide concentration on first segment and tip lengths of *C. parvula* tetrasporophyte branch tips: (5a) first segment length (FSL) for all treatments on all sampling days, and (5b) tip length (TL) for all treatments on all sampling days. Data points are based on means \pm 2S.E. ($n = 15$). Treatment concentration values refer to added bromide; Sm contains approximately 300 μM Br and NSW contains approximately 700 μM Br (see text). Significant differences described in the text are based on $p < 0.0001$.

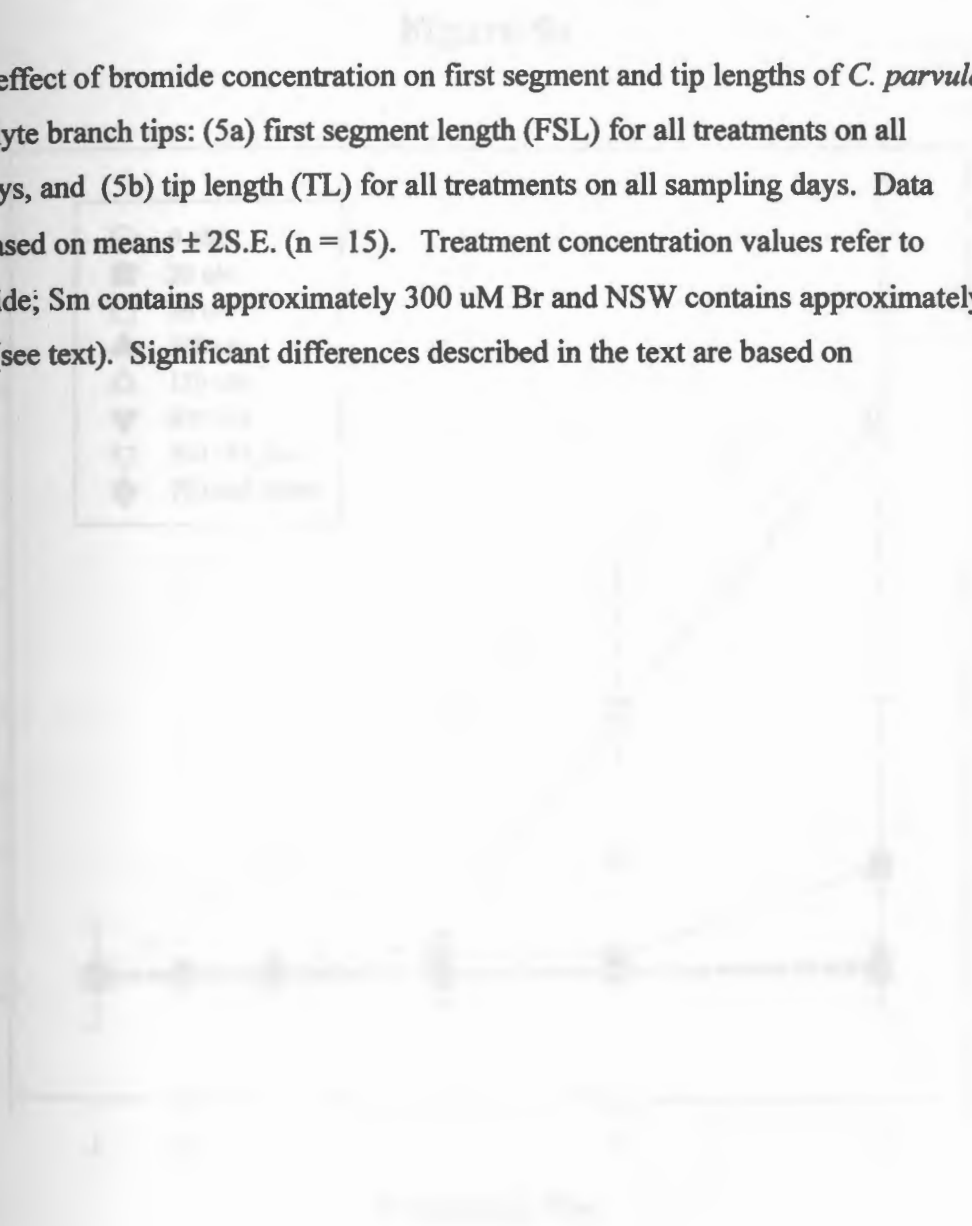


Figure 5a

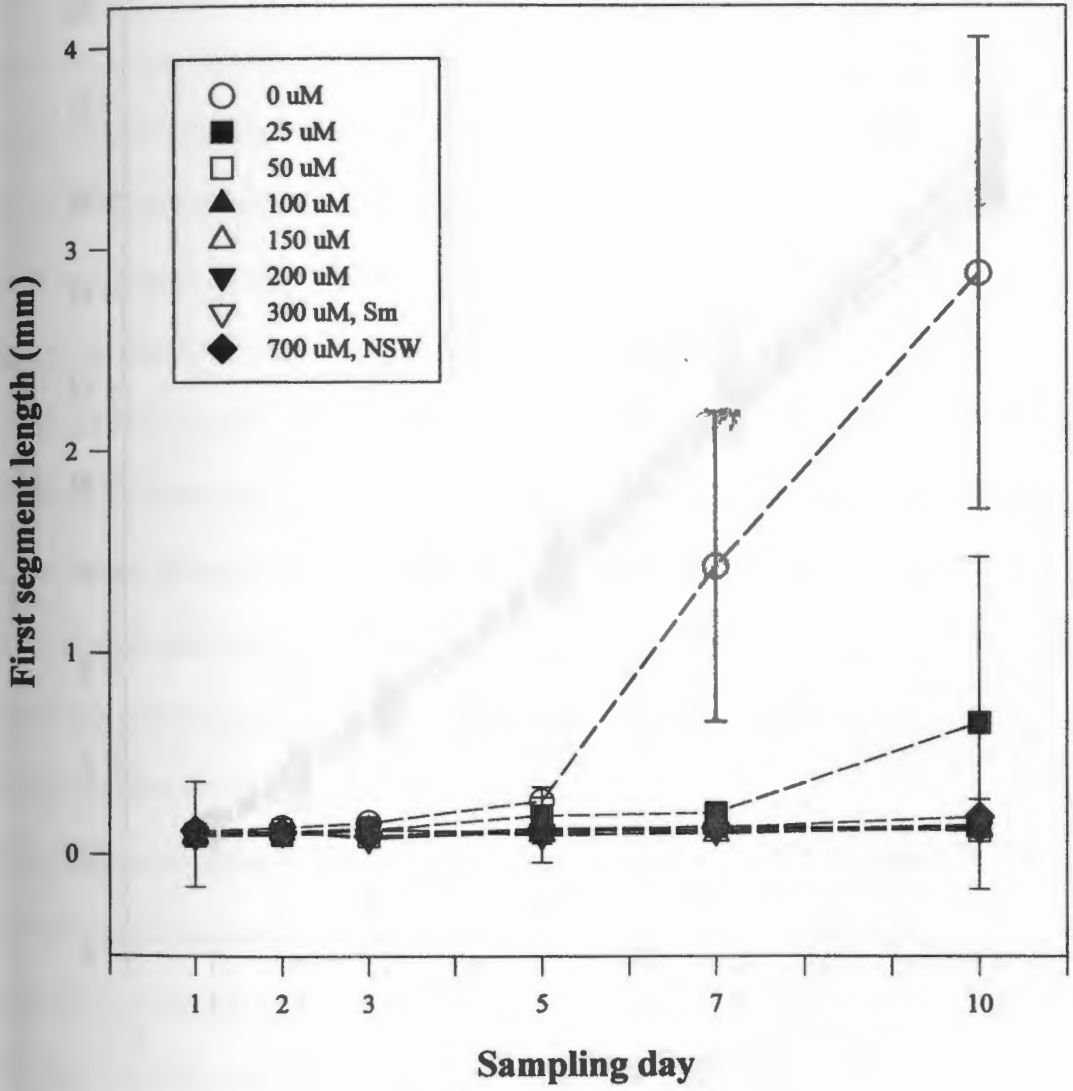
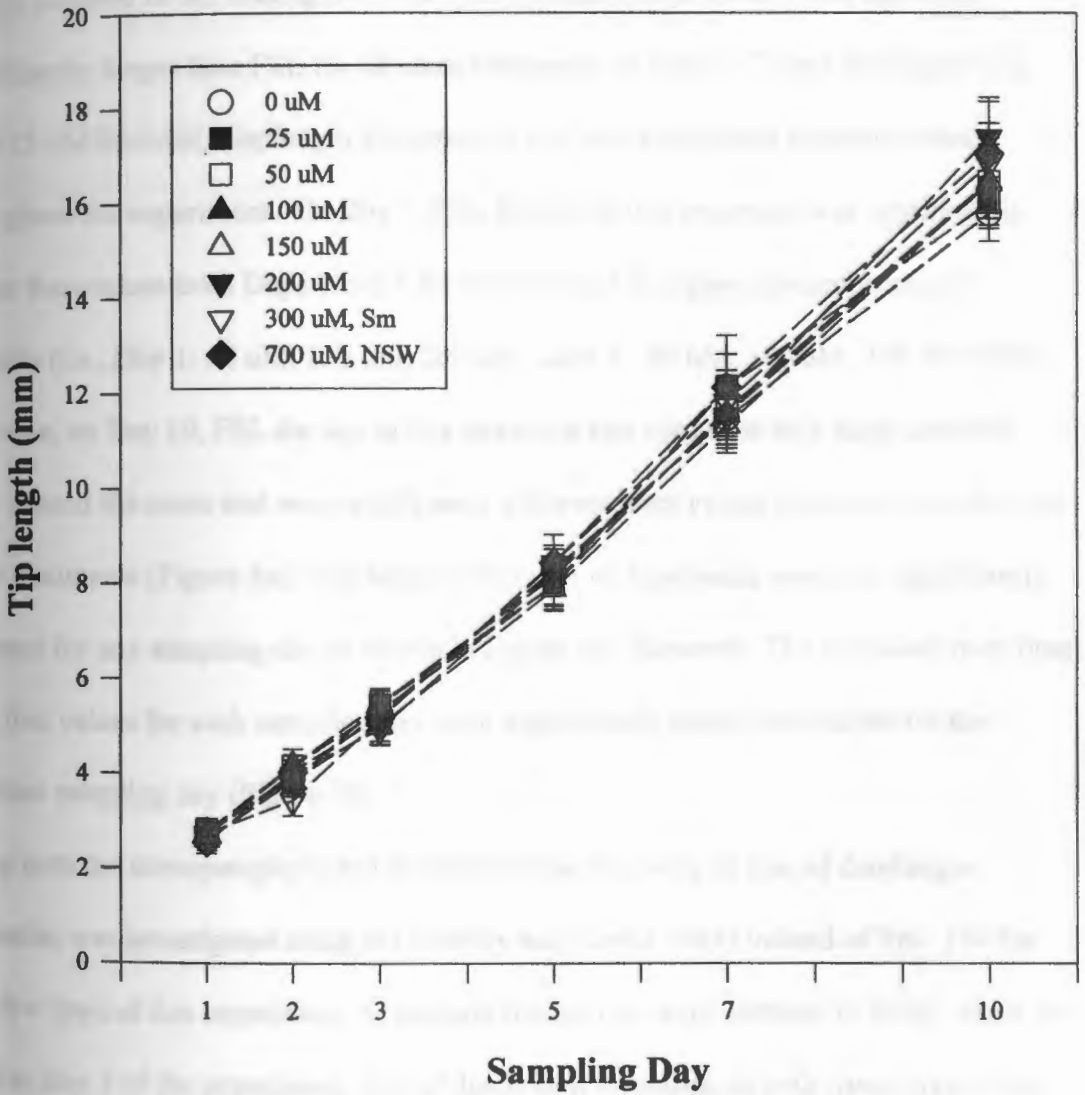


Figure 5b



seawater (Sm). First segment lengths (FSLs) in tips cultured at this and higher concentrations of bromide in Sm were not significantly different from FSLs for tips cultured in NSW (~ 700 μ M bromide) for all sampling days as shown in Figure 5a. FSLs for tips cultured in Sm lacking bromide (0 μ M) ranged from 0.3 to 3 mm and were significantly longer than FSL for all other treatments on Days 5, 7, and 10 (Figure 5a). With 25 μ M bromide, diaphragm formation in tips was maintained to some extent throughout the experiment. On Day 7, FSL for tips in this treatment was significantly longer than values from Days 1 and 3 for tips cultured in higher concentrations of bromide (*i.e.*, Day 1: 50 μ M, 150 μ M, 200 μ M; Day 3: 50 μ M, 300 μ M, 700 μ M-NSW). Likewise, on Day 10, FSL for tips in this treatment had comparatively large standard error around the mean and was significantly different from values from previous days for other treatments (Figure 5a). Tip lengths (TLs) for all treatments were not significantly different for any sampling day as shown in Figure 5b. However, TLs increased over time such that values for each sampling day were significantly larger than values for the previous sampling day (Figure 5b).

Using both the tetrasporophyte and female isolates, recovery of loss of diaphragm formation was investigated using St (Thursby and Harlin 1984) instead of Sm. For the first few days of this experiment, *C. parvula* branch tips were cultured in NSW, St, or St-Br. On Day 3 of the experiment, loss of diaphragm formation in both tissue types from the St-Br treatment was evident. On this day, all tips were changed into NSW and monitored during the recovery period. Figure 6a shows that the data for both tissue types corresponded well. However, FSLs for the tetrasporophyte were significantly larger than FSLs for the female tissue on Days 4 and 5, and the female tissue exhibited a faster

Figures 6a and 6b

Testing the reversibility of loss of diaphragm formation

First segment length (6a) and tip length (6b) for tetrasporophyte (T, open symbols) and female (F, filled in symbols) tissue cultured in NSW, St, St-Br for three days followed by recovery from treatment effects in NSW. On Day 3 of the experiment, all tissue was transferred to NSW. Each data point represents a mean \pm 2S.E. ($n = 10$). Significant differences described in the text are based on $p < 0.01$.



Figure 6a

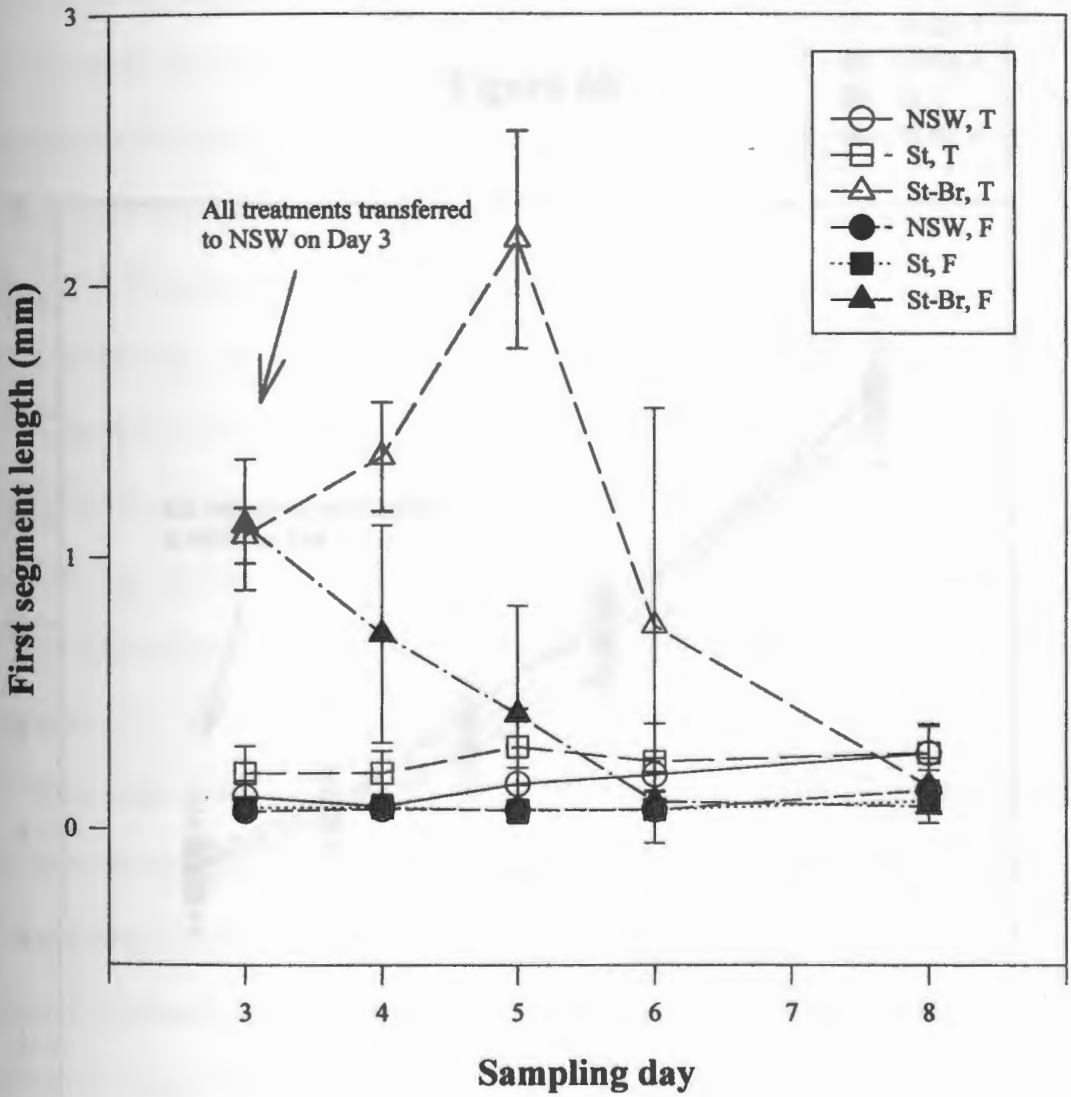
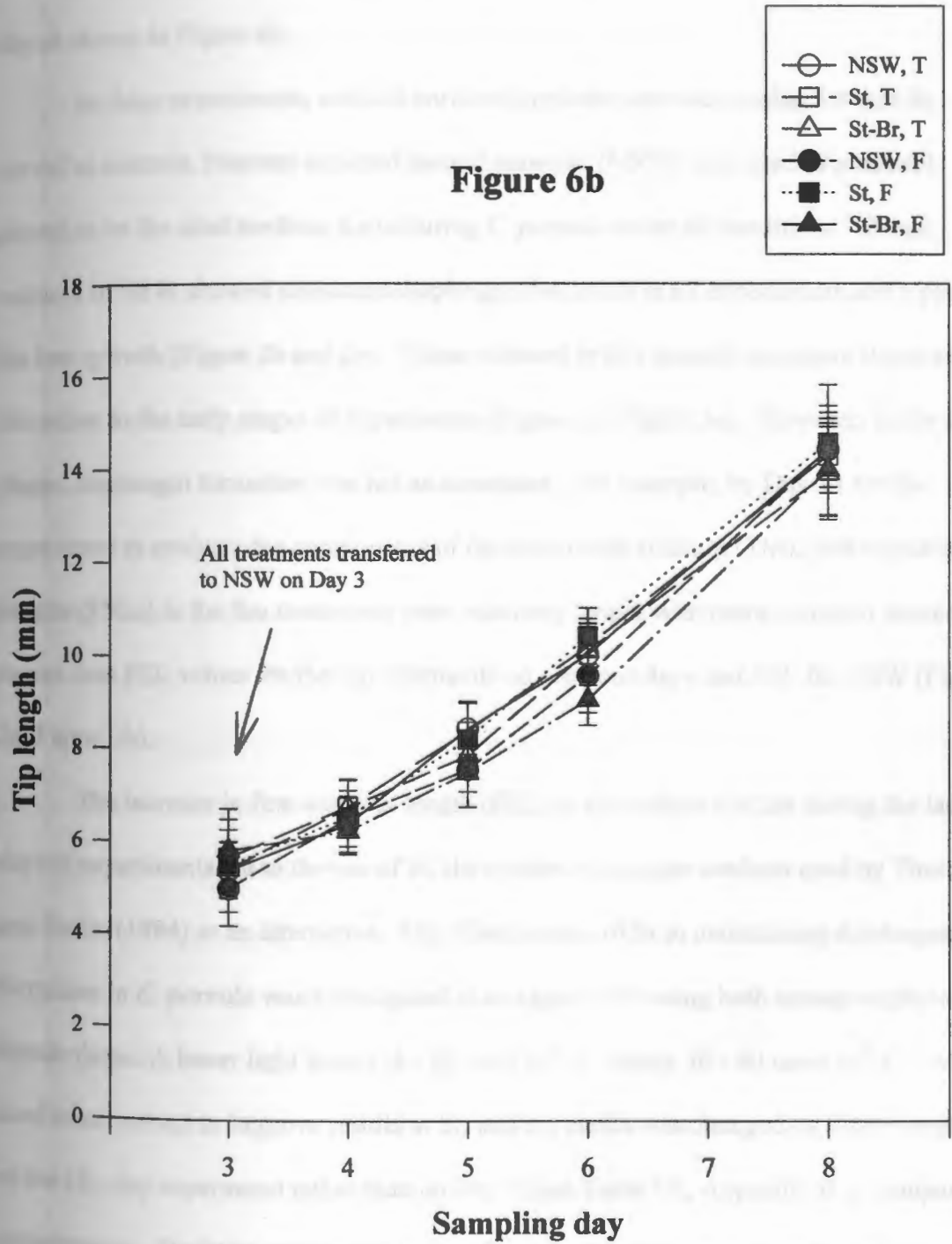


Figure 6b



recovery of diaphragm formation. Tip lengths (TLs) for tetrasporophyte and female tips were not significantly different from each other among treatments within each sampling day as shown in Figure 6b.

In these experiments, nutrient enriched synthetic seawater media, Sm and St, served as controls. Nutrient enriched natural seawater (NSW), also used as a control, proved to be the ideal medium for culturing *C. parvula* under all conditions. Tissue cultured in NSW showed consistent diaphragm formation in all experiments and typically the best growth (Figure 2a and 2b). Tissue cultured in Sm showed consistent diaphragm formation in the early stages of experiments (Figure 3a, Figure 5a). However, in the later stages, diaphragm formation was not as consistent. For example, by Day 14 for the experiment to evaluate the components of the trace metal solution (TM), first segment lengths (FSLs) in the Sm treatments were relatively longer with more variation around means than FSL values for the Sm treatments on previous days and FSL for NSW (Figure 2a, Figure 3b).

The increase in first segment length (FSL) in tips cultured in Sm during the latter days of experiments led to the use of St, the synthetic seawater medium used by Thursby and Harlin (1984) as an alternative. The effectiveness of St in maintaining diaphragm formation in *C. parvula* was investigated in an experiment using both tetrasporophyte and female tissue. A lower light level ($14 - 22 \text{ umol m}^{-2} \text{ s}^{-1}$ versus $30 - 40 \text{ umol m}^{-2} \text{ s}^{-1}$) was used in an attempt to improve results in St, and the media was changed on Days 5 and 10 of the 15 - day experiment rather than on Day 7 (see Table 1A, Appendix B to compare experiments). Statistics were performed on Days 3 - 10 of the experiment using combined data from the tetrasporophyte and female tissue. All significant differences are

based on $p = 0.05$ and $n = 15$. On Day 15 of the experiment, FSLs for St and St-Br were too long to measure easily using image analysis. However, data for NSW was recorded on this day and FSL was measured to be 0.116 ± 0.01 mm (2S.E.).

During the experiment, NSW and St were significantly different from each other for all days except Day 3 (Figure 7). In particular on Days 7 and 10, FSL for St was significantly larger with greater variation around the mean than FSL for NSW. First segment lengths (FSLs) for tips cultured in St-Br were significantly longer than all other treatments within a sampling day on Days 3, 5, 7 and 10 (Figure 7). Data for each sampling day were significantly different from each other with respect to tip length (TL) (data not shown). TL for NSW and St were significantly different only on Day 10. TL in St-Br was significantly shorter than TLs in NSW and St on Day 7. On Day 10, all the treatments were significantly different from each other with TL being longest in NSW and shortest in St-Br.

In a subsequent experiment, the culturing conditions were kept the same but the source of iron in St was varied. The treatments were NSW, St with ferric chloride (StFC; usual source of iron) and St with ferric sulfate (StFS; alternative source of iron). For all sampling days, first segment lengths (FSLs) for tips cultured in StFC and StFS were not significantly different from each other (Figure 8). FSLs for these tips were also not significantly different from FSLs tips cultured in NSW for Days 3, 5, and 7 (Figure 8). On Day 10, however, FSL in NSW was significantly smaller with less variation than for tips cultured in StFC and StFS (Figure 8). On this day, FSLs in these tips was approximately 0.4 mm compared to approximately 0.1 mm for NSW. Increases in tip lengths (TLs) for all three treatments during the experiment were similar (data not

Figure 7

Testing the effects of culture with St, lower light, and frequent media changes

First segment length for the combined data for tetrasporophyte and female tissues cultured in synthetic seawater media (St) at lower level ($14 - 22 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$) and with media changes every five days. Treatments included NSW, and St and St-Br. Each data point represents a mean \pm 2S.E. ($n = 30$) for Days 3, 5, 7 and 10 of the experiment. Significant differences described in the text are based on $p < 0.0001$.

Figure 7

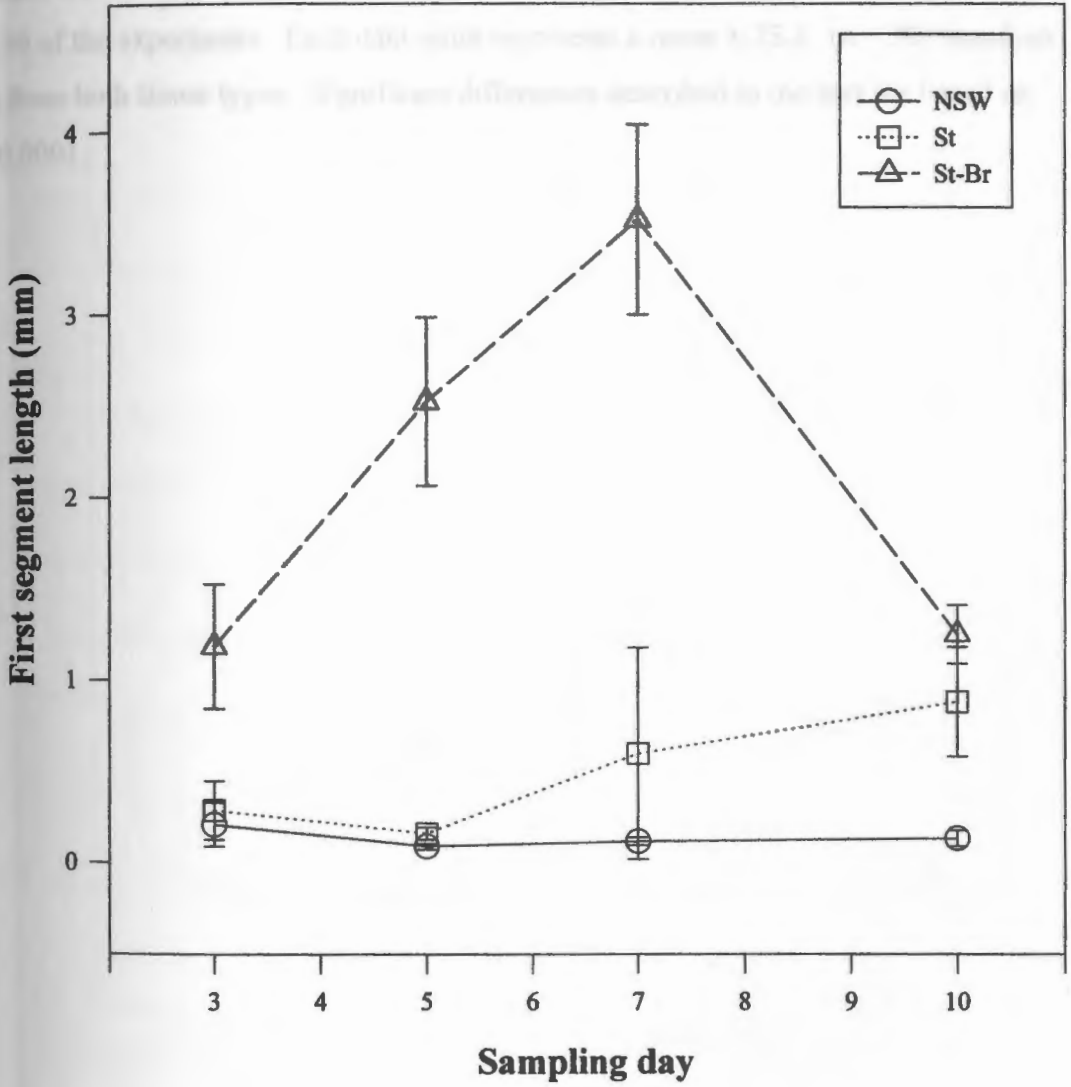


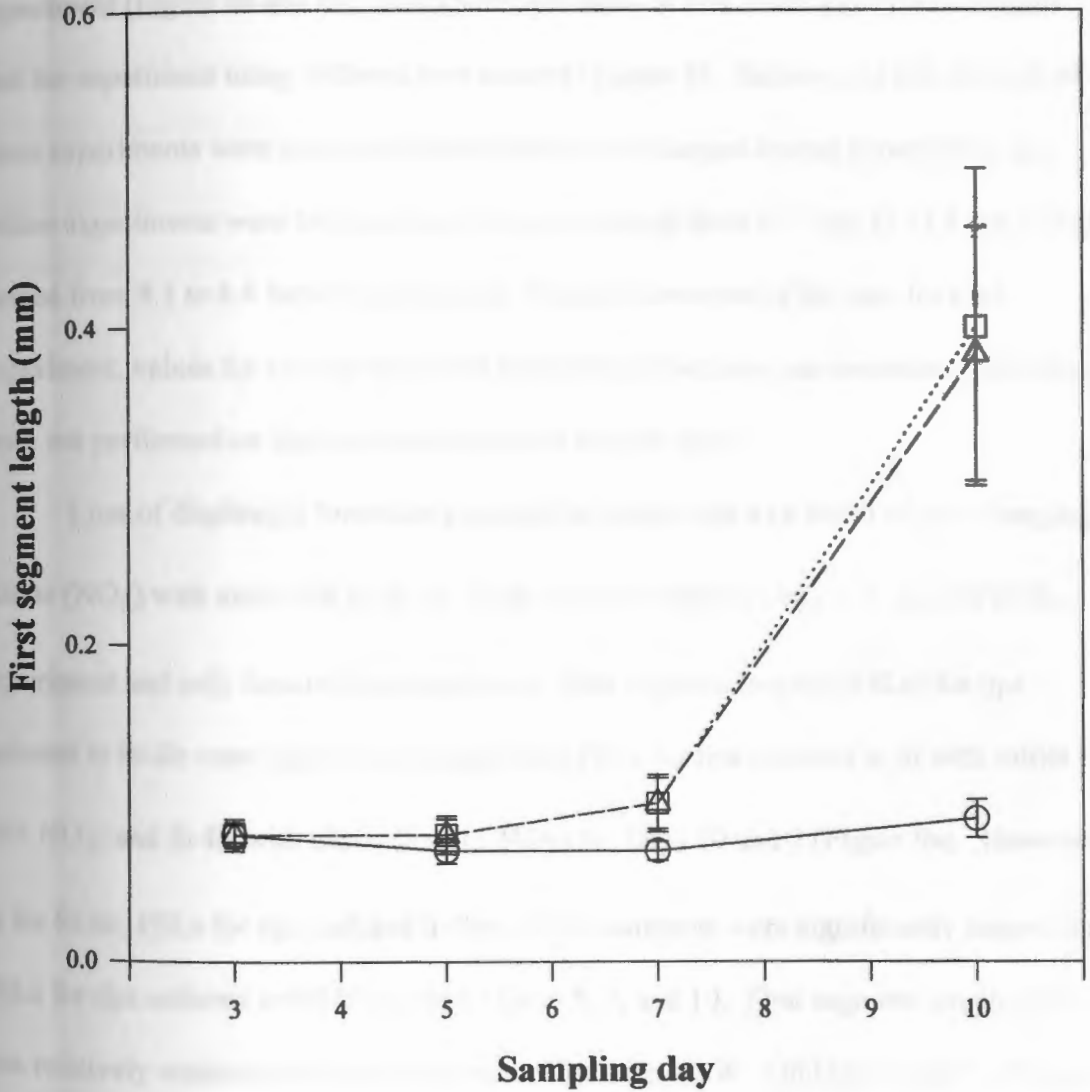
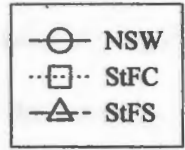
Figure 8

Testing the effect of ferric chloride versus ferric sulfate in St

First segment length for tetrasporophyte and female isolates cultured in synthetic seawater media with ferric chloride (StFC) and with ferric sulfate (StFS) for Days 3, 5, 7 and 10 of the experiment. Each data point represents a mean \pm 2S.E. (n = 30) based on data from both tissue types. Significant differences described in the text are based on $p < 0.0001$.



Figure 8



shown). TLs within sampling days were not significantly different, but TLs for a given sampling day was significantly larger than TLs for the previous sampling day.

In order to exclude changes in salinity and pH as causes for the variability in FSL for tips cultured in synthetic media, these parameters were examined for the recovery experiment (Figure 6a and 6b), the experiment using St and lower light levels (Figure 7), and the experiment using different iron sources (Figure 8). Salinity and pH for each of these experiments were measured before media was changed during experiments and before experiments were broken down. Salinity ranged from 29.7 ppt to 31.5 ppt and pH ranged from 8.1 to 8.6 for all experiments. From observation of the data for each experiment, values for salinity or pH did not distinguished any one treatment. Statistics were not performed on the data due to unequal sample sizes.

Loss of diaphragm formation occurred in branch tips as a result of interchanging nitrate (NO_3) with nitrite (NO_2) in St. Data was collected on Days 5, 7, and 10 of the experiment and only female tissue was used. First segment lengths (FSLs) for tips cultured in St-Br were significantly larger than FSLs for tips cultured in St with nitrite ($\text{St} + \text{NO}_2$) and St-Br with nitrite ($\text{St-Br} + \text{NO}_2$) for Days 10 and 7 (Figure 9a). However, as for St-Br, FSLs for tips cultured in the nitrite treatments were significantly larger than FSLs for tips cultured in NSW and St on Days 5, 7, and 10. First segment length (FSL) was relatively constant over time for tissue cultured in NSW. On Days 5 and 7, FSL in St was indistinguishable from FSL in NSW, however, on Day 10 FSL in St approached 1.0 mm and was significantly larger than FSL for tips cultured in NSW. By comparison, FSLs for tissue cultured in $\text{Sm} + \text{NO}_2$, Sm-Br , and $\text{Sm-Br} + \text{NO}_2$, were near or greater than

Figures 9a, 9b and 9c

The effects of replacing nitrate with nitrite in synthetic seawater media with and without added bromide

First segment length (FSL; 9a), cuticle thickness (TL; 9b), and number of trichogynes (9c) for Days 5, 7, and 10 of an experiment using female tissue. Each data point represents a mean \pm 2S.E. ($n = 5$). Treatments included: NSW, St, St with nitrate replaced with nitrite (St+NO₂), St minus bromide (St-Br), and St minus bromide with nitrate replaced with nitrite (St-Br+NO₂). Significant differences described in the text are based on $p < 0.0001$.



Figure 9a

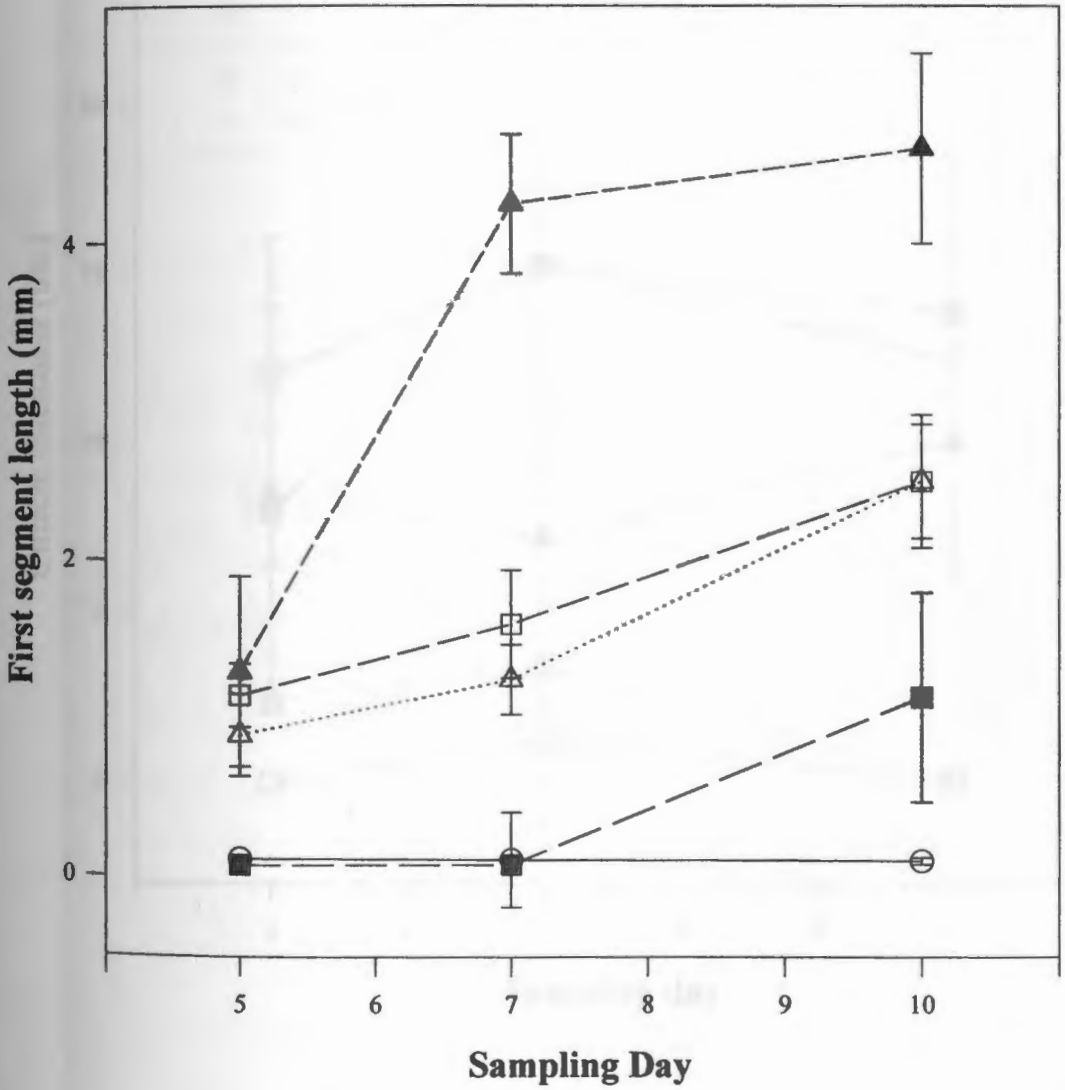
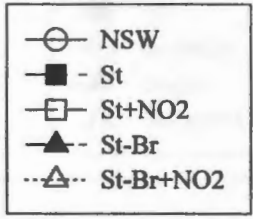


Figure 9b

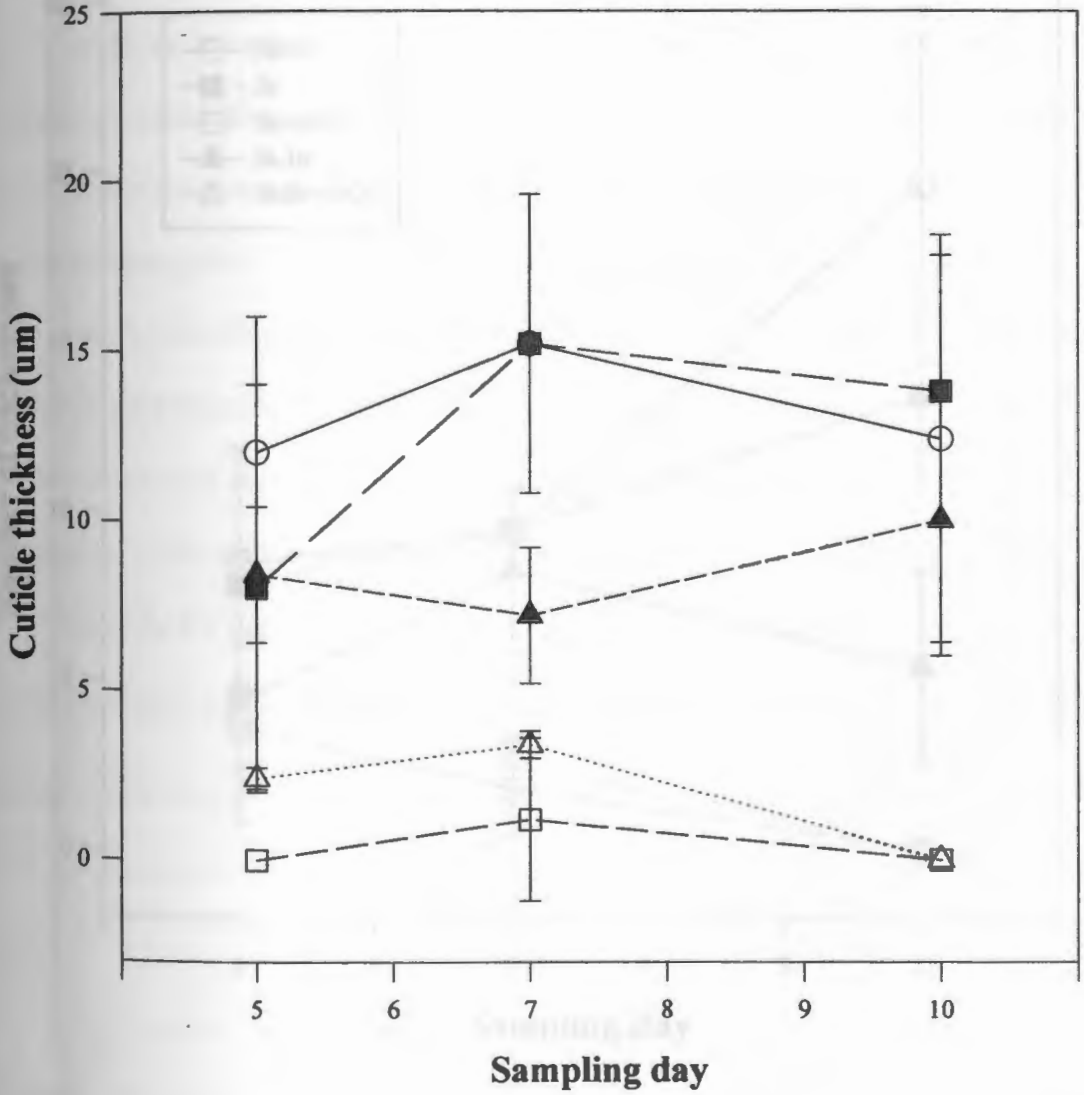
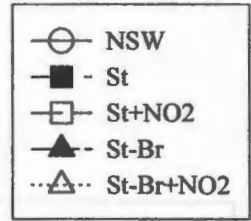
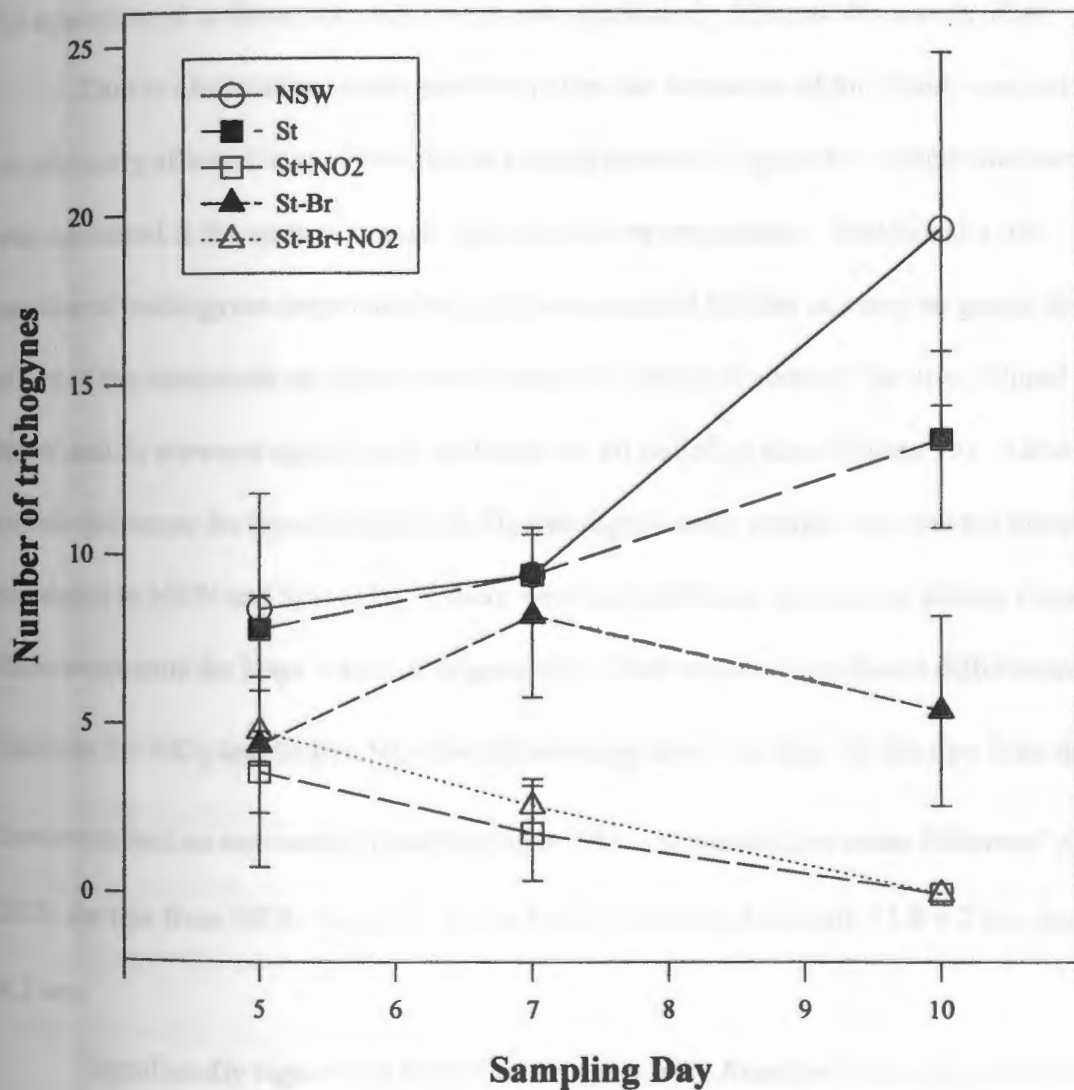


Figure 9c



1.0 mm on Days 5, 7, and 10. With regard to tip length (TL), tips cultured in both St+NO₂ and St-Br+NO₂ were significantly smaller than tips cultured in NSW, St, and St-Br for Days 5, 7 and 10 (data not shown). On Day 10 only, TL for St-Br was significantly smaller than TLs for NSW and St, and TL for St was smaller than NSW. Otherwise, TLs for tips cultured in these treatments were not significantly different from each other.

Due to observations made previously that the formation of the cuticle seemed to be adversely affected in synthetic media lacking bromide (Figure 4a), cuticle thickness was measured at the apex of branch tips in the nitrite experiment. Additionally, the number of trichogynes (reproductive hairs) was counted for tips as a way to gauge the effect of the treatments on reproductive capacity. Cuticle thickness' for tips cultured in NSW and St were not significantly different for all sampling days (Figure 9b). Although cuticle thickness for tips cultured in St-Br was significantly smaller than the thickness' measured in NSW and St on Day 7, there were no significant differences among these three treatments for Days 5 and 10 (Figure 9b). There were no significant differences between St+NO₂ and St-Br+NO₂ for all sampling days. On Day 10, the tips from these treatments had no measurable cuticles (Figure 9b). In contrast, the mean thickness' \pm 2S.E. for tips from NSW, St and St-Br for Day 10 were 12.4 ± 3 μ m, 13.8 ± 2 μ m, and 10 ± 2 μ m.

Significantly higher numbers of trichogynes were found in branch tips cultured in NSW and St compared to the other treatments for Day 10. However, the mean number for NSW 19.6 trichogynes was significantly higher than the mean for St, 13.4 trichogynes for this day (Figure 9c). On Days 5 and 7, the values for NSW and St did not differ significantly. Trichogynes were very few (Day 7) or nonexistent (Day 10) for tips

cultured in St+ NO₂ and St-Br+ NO₂ (Figure 9c). The number of trichogynes in these treatments was significantly lower than for tips cultured St-Br for Day 10 (Figure 9c).

Reproduction in NSW and Sm was investigated in three non-replicate tests (Table 2). Both cystocarps and spores were produced in NSW and Sm (tests #1 and #2). However, cystocarps were generally smaller on plants cultured in Sm than in NSW. Additionally, based on observations of settled spores on the bottoms of culture flasks, the number of spores produced in NSW seemed greater than the number of spores produced in Sm. When spores were allowed to continue to develop in the absence of adult plants and in the medium in which they originated, 1.7 g and 4 mg of germling material was collected in NSW, and 1.0 g and 3.5 mg of germling material was collected in Sm for tests #1 and #2, respectively. Tetraspores were not present on germlings collected from NSW or Sm; however, while germlings from NSW had diaphragms, germlings from Sm lacked diaphragms.

At the end of test #3, plants grown in NSW had vase-shaped cystocarps and had produced many spores (as was evident on the bottom of the flask). Although, cystocarps were evident in Sm, settled spores on the bottom of the culture flask were not visible. Additionally, it was noted the first segment length (FSL) in this tissue was relatively long compared to FSL for tissue grown in NSW. Cystocarps and spores were not produced by tissue cultured in Sm-Br.

Growth of tetrasporophyte and female tissue in NSW, Sm, and Sm-Br was investigated (data not shown). Tissue was examined for each treatment on the day the media was changed and at the end of the culturing period. In each run of the experiment (n = 9), diaphragm formation was continuous in NSW; microscopic observation revealed

Table 2

Data from three nonreplicate tests on cystocarp and carpospore production by female tissue cultured in NSW and Sm

Test	Duration of test (Days)	Media changed (Day)	Appearance of cystocarps (Day)	Appearance of spores (Day)	End weight (g)	Number of cystocarps for 5 plants
#1	25	15				
NS W			15	16	3.62	16, 16, 13, 6, 12
Sm			15	18	3.63	7, 12, 12, 11, 12
#2	25	7				
NS W			13	16	1.29	17, 11, 11, 13, 12
Sm			13	19	1.25	6, 10, 15, 7, 11
#3	17	7				
NS W			?	13	no data	no data
Sm			?	13	“ “	“ “
Sm- Br			none	None	“ “	“ “

that first segment lengths (FSLs) were relatively small (no measurements were made) and that diaphragms were regularly spaced on branch tips. However, in Sm, diaphragms were not as closely spaced as they were in the tissue cultured in NSW. In Sm-Br, it was noted during media changes that diaphragm formation in branch tips had ceased and the apical regions of the tips were malformed. By the end of each run (ranging from Day 10 to Day 20), diaphragm formation had ceased in both Sm and Sm-Br. The misshapen appearance of branch tips of tissue cultured in Sm-Br, was less frequently seen in Sm. Significant differences ($p = 0.05$) in the mean weight of tissue ($\pm 2S.E.$) collected occurred between the female tissue cultured in NSW (4.30 ± 1.0 g) and tetrasporophyte tissue cultured in Sm-Br (1.25 ± 0.6 g). When the data for female and tetrasporophyte tissue were combined, significant differences in the mean mass of tissue collected occurred between tissue cultured in NSW (3.5 ± 1.2 g) and Sm-Br (1.44 ± 0.46 g).

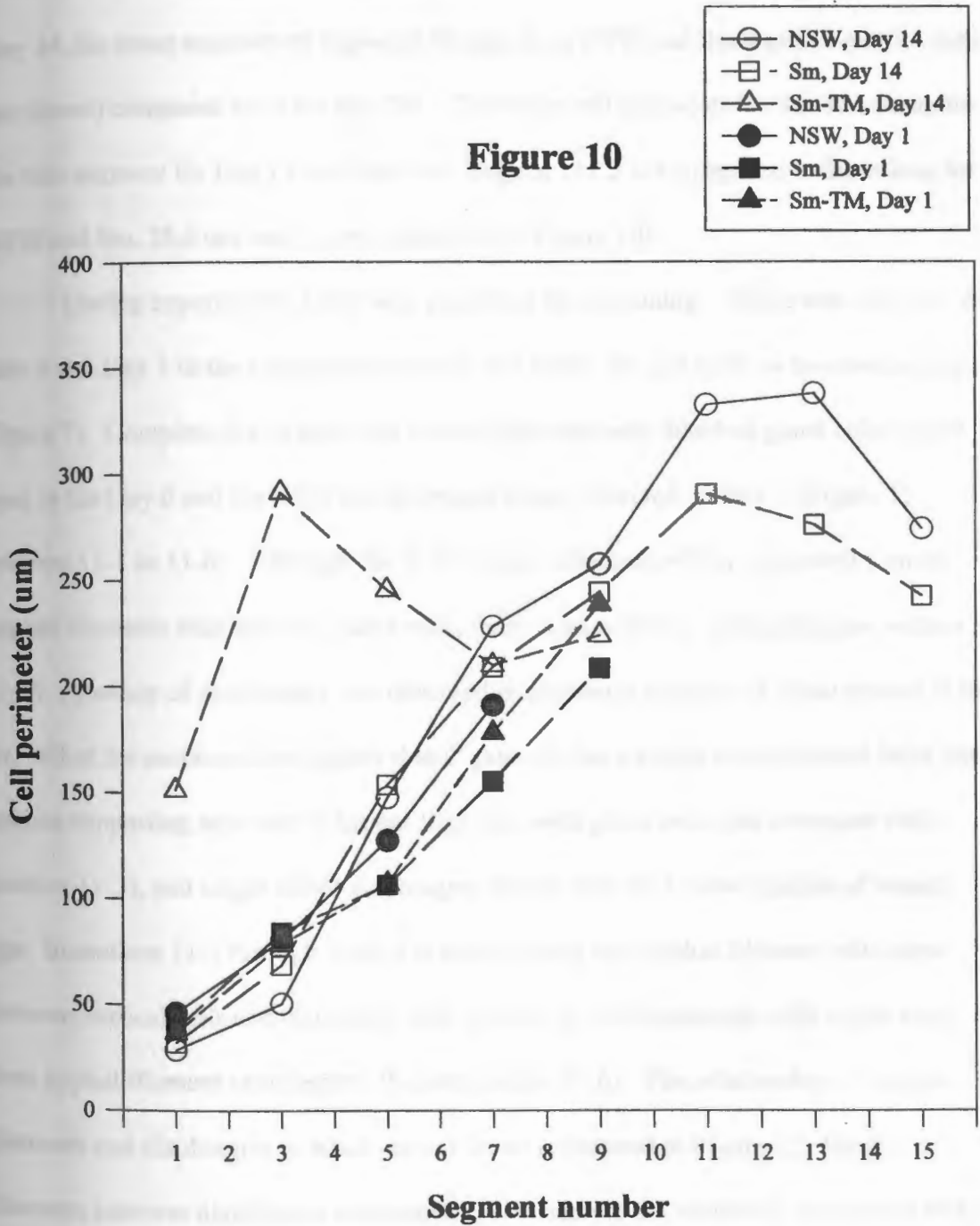
In an extended investigation of growth of *C. parvula*, data for mean cell perimeter per segment from the early experiment (see first paragraph in Results) was plotted. For all sampling days in this experiment (1, 2, 3, 5, 7 and 14), mean cell perimeters for NSW and Sm were similar to each other for corresponding segments (Figure 10; data not shown for Days 2, 3, 5 and 7). In both treatments, mean cell perimeters increased with the segment number. In other words, cells expanded with age as their place along the branch axis increased in distance from the apex due to new apical growth and new diaphragm, and therefore, segment formation. By comparing the data points for all treatments for Day 1 (filled in symbols) to the data points for Sm-TM, it is evident that

Figure 10

Cell perimeters per segment for tetrasporophyte tissue cultured in NSW, Sm, and Sm-TM

Cell perimeters for each segment were measured for tetrasporophyte tissue in the early experiment (see first paragraph of Results). Means for Day 1 (filled symbols) are compared to means for Day 14 (open symbols). Each data point represents the mean perimeter of 18 cells (6 cells were measured from three branch tips collected from one flask per sample day).

Figure 10



few new segments have formed in the Sm-TM branch tips. In contrast, tips from the NSW and Sm treatments continued to form new segments during the experiment. By Day 14, the mean numbers of segments for tips from NSW and Sm were 18 and 19 (data not shown) compared to 11 for Sm-TM. The mean cell perimeter for Sm-TM tissue for the first segment for Day 14 was relatively large at 151.2 μm compared to the values for NSW and Sm, 28.6 μm and 32 μm , respectively (Figure 10).

During experiments, tissue was preserved for sectioning. Tissue was collected on Day 0 and Day 3 in the experiment using St and NSW, St, and St-Br as treatments (see Figure 7). Complete diaphragms and hyphal filaments with attached gland cells can be seen in the Day 0 and the NSW and St treated tissue collected on Day 3 (Figure 11, sections 11-1 to 11-6). Although the St-Br tissue collected on Day 3 has well formed hyphal filaments with attached gland cells, there is no evidence of diaphragms (section 11-7). Absence of diaphragms was observed in numerous sections of tissue treated in St-Br. All of the sections show clearly that *C. parvula* has a single celled cortical layer, an interior supporting structure of hyphal filaments with gland cells and connector cells (section 11-3), and single celled diaphragms which span the hollow interior of branch tips. In sections 11-1 through 11-6, it is worth noting that hyphal filament cells come between cortical cells and diaphragm cells indicating that diaphragm cells might arise from hyphal filament cells (especially note section 11-6). The relationship of hyphal filaments and diaphragms in whole mount tissue is featured in Figure 12. Hyphal filaments intersect diaphragms unchanged (12-1) and yet are intimately associated with diaphragms (12-1, 12-2, and 12-3). The ends of a hyphal filament cell were observed to

Figure 11

Sections of branch tips treated in NSW, Sm and Sm-Br

Brightfield micrographs of sections of female branch tips from the experiment featured in Figure 7. Sections represent female tissue on Day 0 (1 & 2) and tissue from the NSW (3 & 4), Sm (5 & 6), and Sm-Br (7) treatments on Day 3 of the experiment. Symbols: diaphragms (arrow), hyphal filaments (arrowhead), and gland cell (double arrows). The magnification of sections 1, 3, 5 and 7 is 60X, and 120X for the remaining sections.



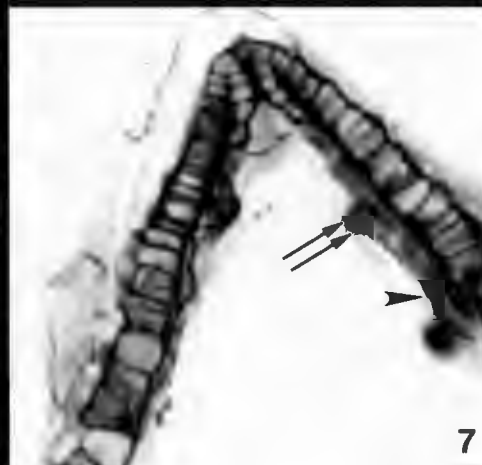
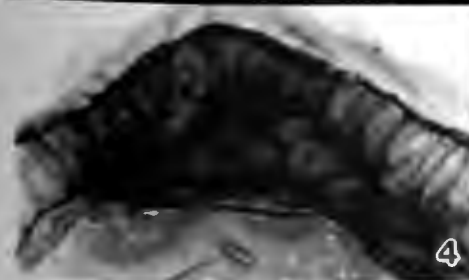
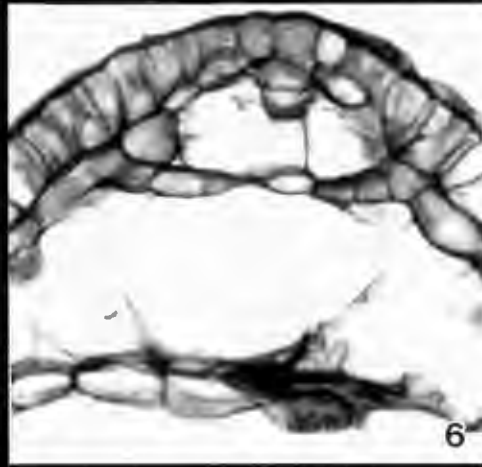
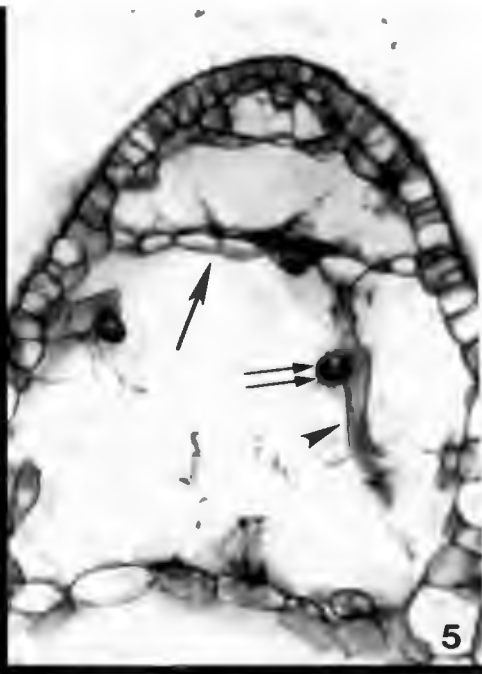
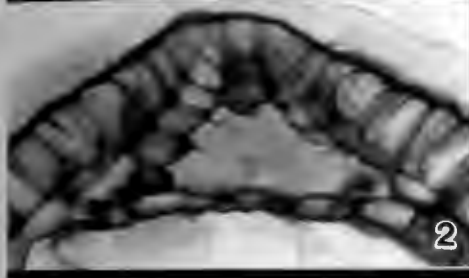
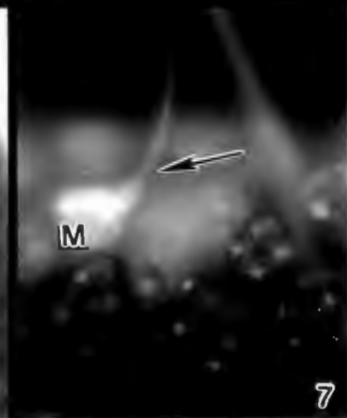
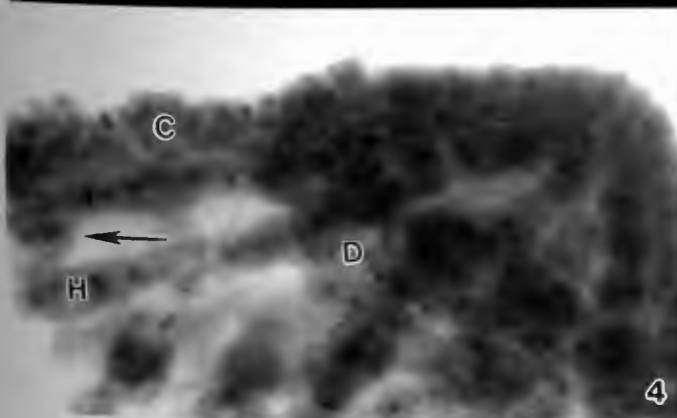
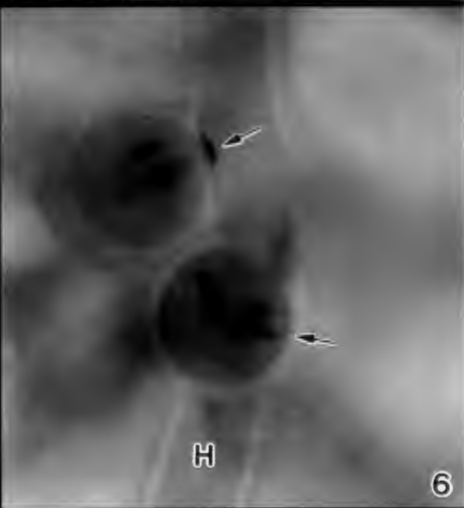
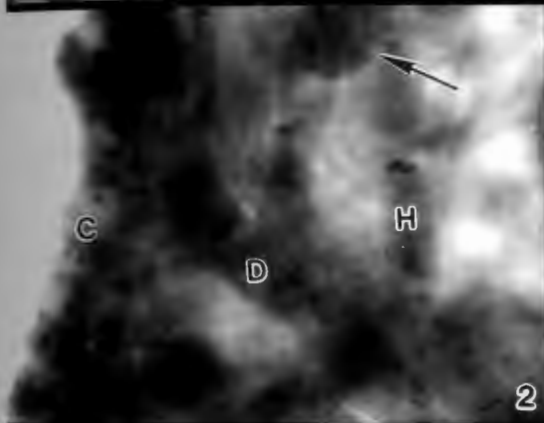
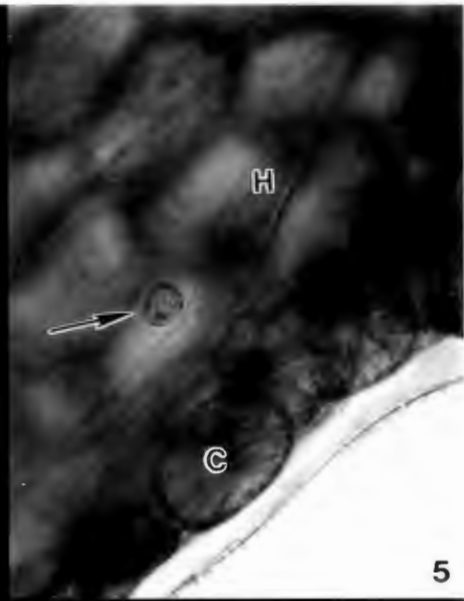
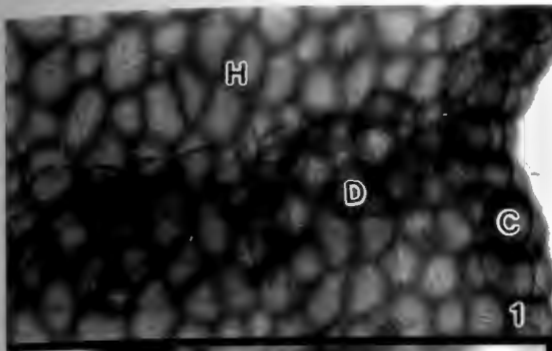


Figure 12

Brightfield and fluorescent micrographs of whole mount, Wittman stained and DAPI stained tissue

Descriptions of micrographs by number: (1) Hyphal filaments intersecting the diaphragm of a female branch tip, 27X; (2) Wittman's stain highlights the nuclei in cortical cells, diaphragm cells, and hyphal filament cells as they intersect the diaphragm, 54X; (3) DAPI stain faintly identifies nuclei at the apex of a branch tip cultured in St-Br; hyphal filaments with gland cells radiate from the apex, 54X; (4) Wittman's stain highlights the nuclei of cortical cells, hyphal filament cells, gland cells, and cells of the developing diaphragm, 108X; (5) The attachment of a hyphal filament to the cortical wall is directly opposite the filament's gland cells, a connector cell is not apparent, 54X; (6) A hyphal filament and gland cells stained with Wittman's stain; chromosomes of the gland cells are visible and the attachment point of the gland cell is stained, 162X; (7) DAPI stained mother cell (M) to a sterile hair (arrow), 120X. Abbreviations: D = diaphragm cells, H = hyphal filament cells, C = cortical cells, arrow = gland cell.



fall above (12-2) and below the diaphragm through which the cell was passing (Figure 11).

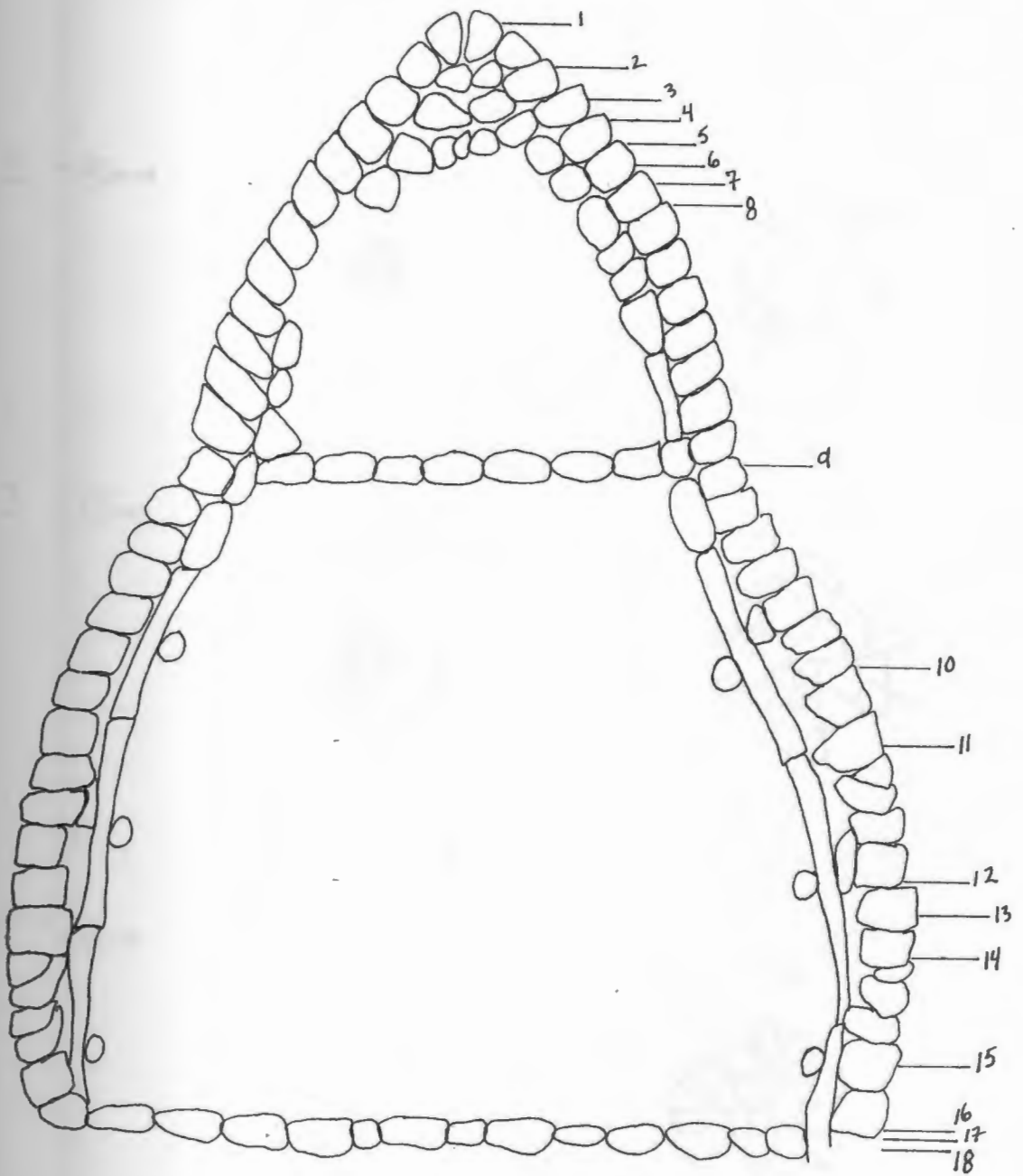
The histology on cultured *C. parvula* was supported in the literature and through observation of *C. parvula* collected from the wild (Figure 13a and 13b). Figure 13a is based both on the work of Bigelow (1887) and Davis (1892) and on the observations made of the sections described above. The branch tip in the figure was drawn to represent a 0.34 mm branch tip with the first measurable segment (using image analysis) at 0.14 cm. A developing segment, as seen at the apex of the diagram, would be measured at 0.04 mm as the first segment if the diaphragm was clearly delimited (Figure 13a). The markings numbered 1 - 18 are the hypothetical placements for the cross sections taken from a female branch tip collected from Sheep's Point Cove, Newport, Rhode Island (Figure 13b). The serial sections, drawn using a camera lucida, represent 5 μ m intervals along the branch tip; however, it is likely that some sections may have been lost during sectioning resulting in gaps in the collection.

The apical cells of the branch tip compose section #1 and possibly section #2. In sections #3 and #4, about 20 μ m from the apex, cells are grouped into six wedges forming what appears to be a developing diaphragm (Figure 13b). In section #5, seven hyphal filament cells surrounding a hollow interior are distinguishable from the outermost, smaller cortical cells (Figure 13b; the hyphal filament cells are separated from the cortical cells in the drawings). These same cells appear slightly larger in sections #6 and #7. In section #8, nine hyphal cells are present and the cortical layer is nearly one cell layer thick. Loosely ordered wedges of cells, possibly forming the first measurable

Figures 13a and 13b

Schematic diagram and traced cross sections of a *C. parvula* branch tip

The diagram (13a) is based on the work of Bigelow (1887), Davis (1892) and on personal observations of sections and whole tissue. Scale: 5 μ m = 0.1 mm. The markings 1 - 18 are the hypothetical placements for the cross sections taken from a female branch tip collected from the wild (13b). The serial sections approximate 5 μ m intervals along the branch tip; however it is likely that this series is incomplete due to having lost some sections during processing. Details about sections by number: 1. free-hand drawing using camera lucida, 2 - 4. left hand drawings are traces using the camera lucida, right hand drawings were done free-hand using the camera lucida as a guide, 5. all drawings were done using the camera lucida with the far left drawing showing all cells, the middle drawing showing only the interior cells, and the far right showing only the cortical, outermost cells, 6 - 18. all drawings were done using the camera lucida with left hand drawings showing the interior cells of the thallus and right hand drawings showing the cortical, outermost cells of the thallus. The dot above each section is a reference point. Abbreviations: H = hyphal filament cell, # = gland cell, * = connector cell. Scale: 2 cm = 0.1 mm.



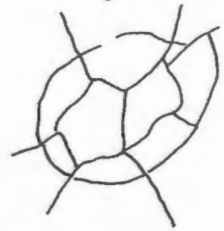
5cm = 0.1mm

A.

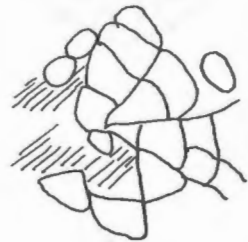
1. $\sim 5\mu\text{m}$



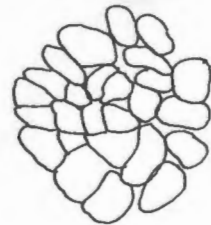
2. $\sim 10\mu\text{m}$



3. $\sim 15\mu\text{m}$



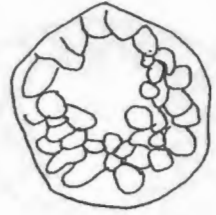
4. $\sim 20\mu\text{m}$



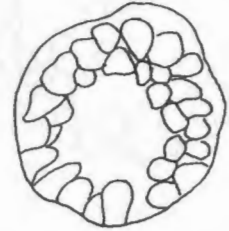
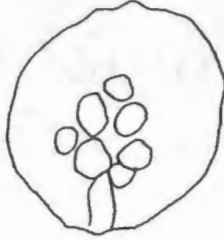
B.

$$\underline{2\text{cm}} = 0.1\text{mm}$$

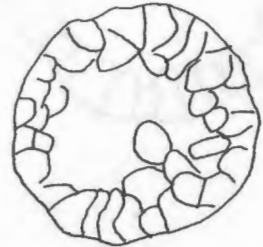
5. ~25 μ m



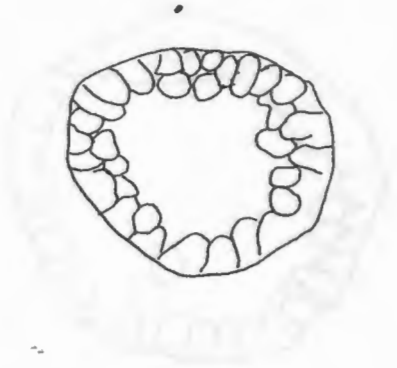
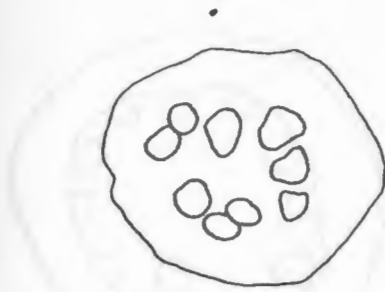
6. ~30 μ m



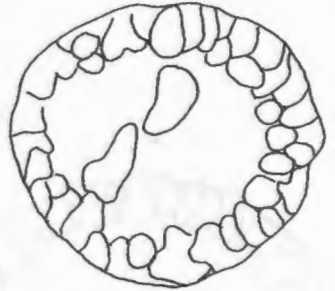
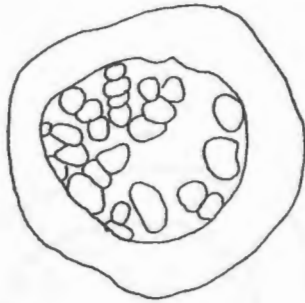
7. ~40 μ m



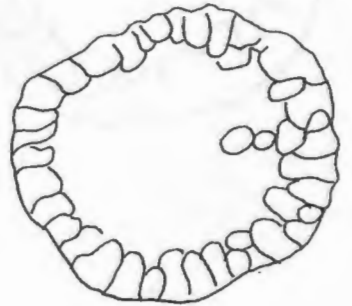
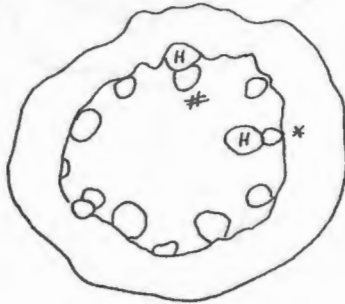
8. ~45 μ m



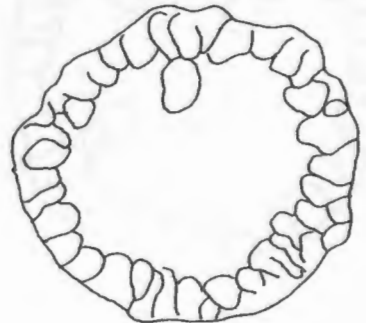
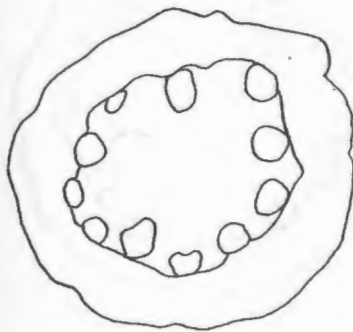
9. ~50 μ m



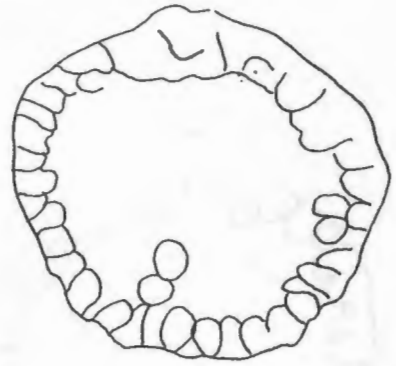
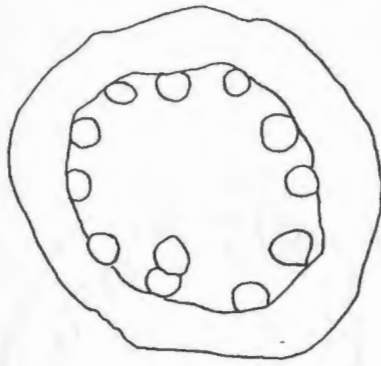
10. ~55 μ m



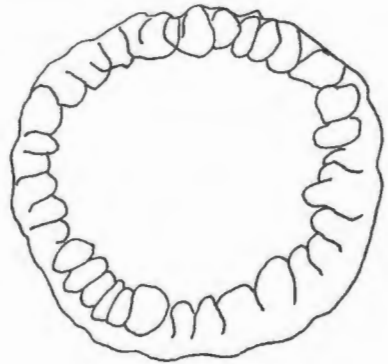
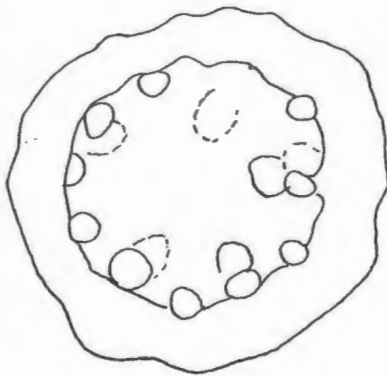
11. ~60 μ m



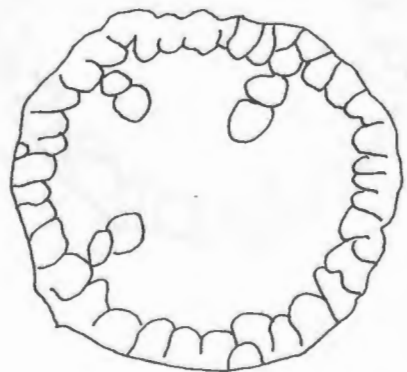
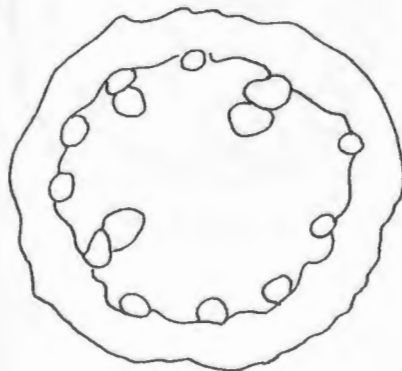
12. ~65 μ m



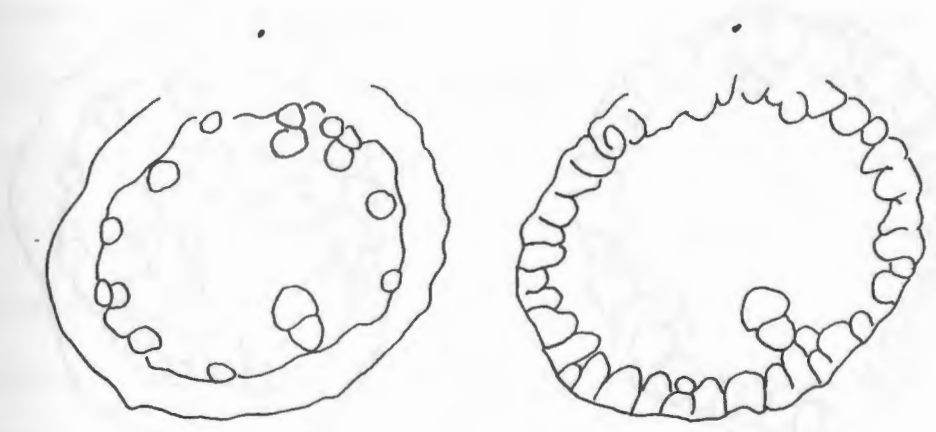
13. ~70 μ m



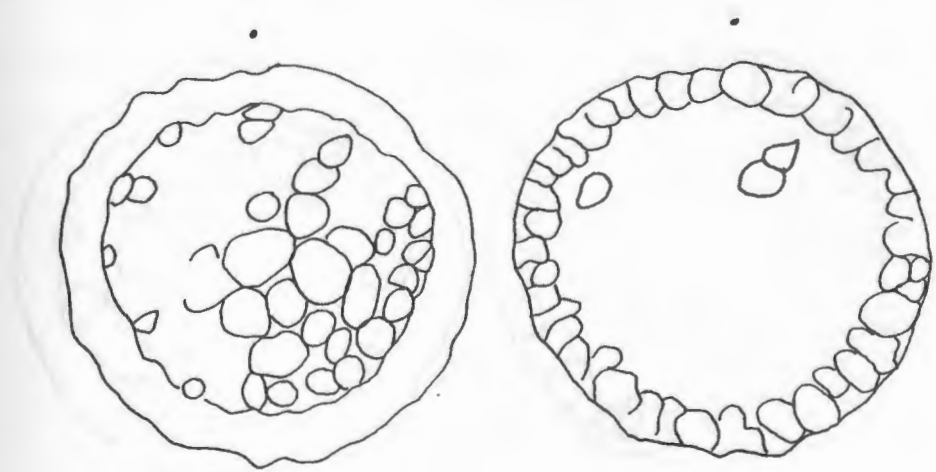
14. ~75 μ m



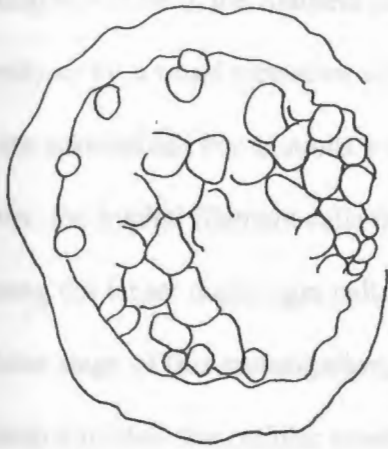
15. ~ 80 μ m



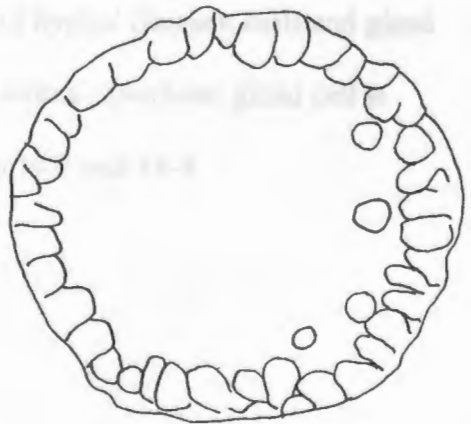
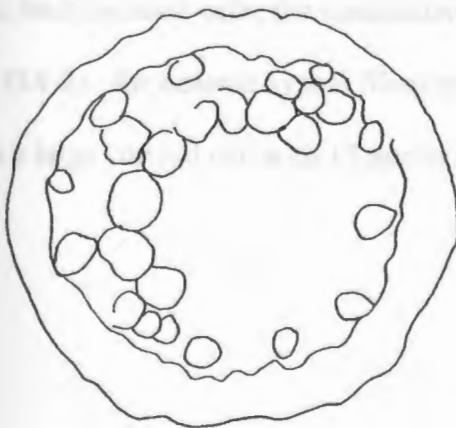
16. ~ 85 μ m



17. ~90 μ m



18. ~95 μ m



diaphragm, are seen in section #9. The first evidence that hyphal filaments have divided to form gland cells and connector cells is in section #10 (about 55 um from the apex). According to Bigelow (1887), the filament connects to the cortical wall opposite the gland cell directly or by a small connector cell. For sections #11 - 15, eleven hyphal filament cells are noticeable. For sections #16 - 18, a second complete diaphragm is present, however, the hyphal filament cells that are a part of this diaphragm are hard to distinguish among the larger diaphragm cells.

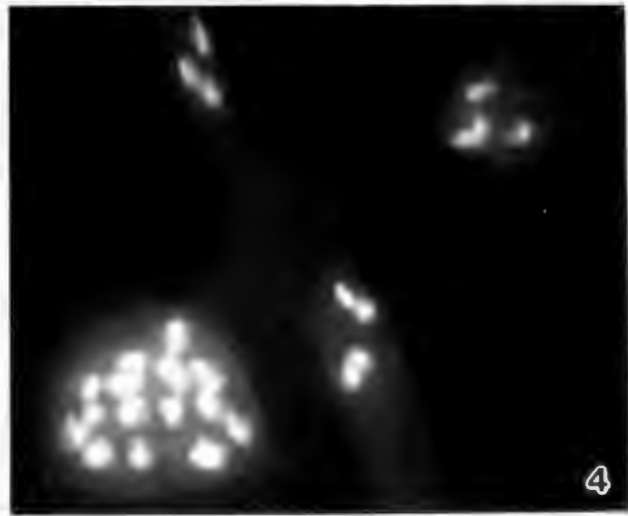
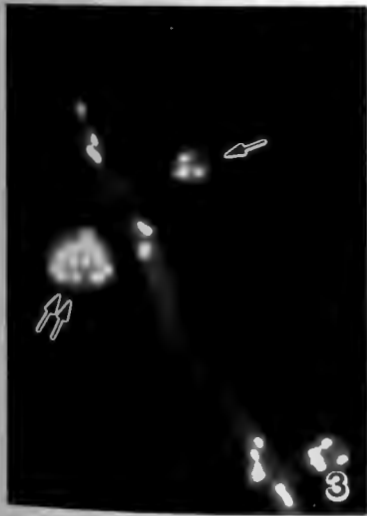
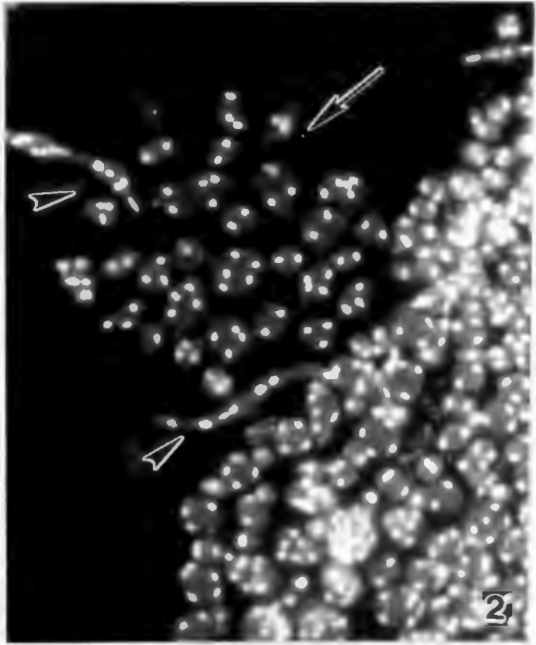
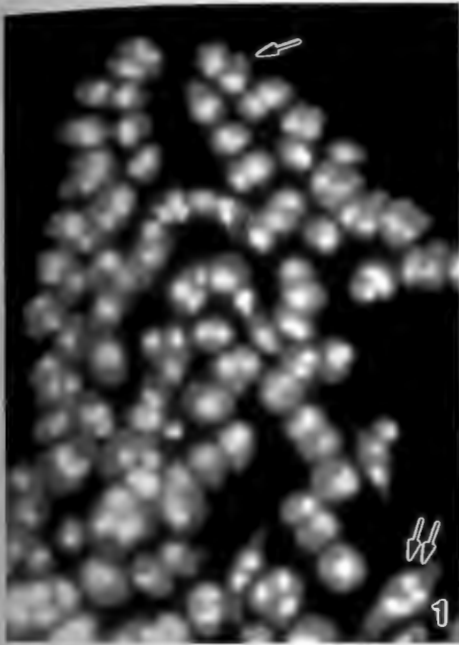
In the later stage of this investigation, tissue squashes were done to see if the results would help elucidate the cellular events associated with diaphragm formation. The micrographs in Figure 14 are of healthy female tissue. The squashed tissue in this experiment facilitated the identification of cell lineage and associations. For example, uninucleate cells are found at the apex of a branch tip. These cells undergo anticlinal divisions and, eventually, may divide in a number of planes as tissue ages and cells increase in size and number of nuclei (14-1). A diaphragm plate is composed of relatively large, multinucleate cells; the association of hyphal filament cells and gland cells is evident (14-2). An isolated hyphal filament with a trinucleate gland cell is associated with a large cortical cell with 15 nuclei in 14-3 and 14-4.

Figure 14

Fluorescent micrographs of fixed and squashed, DAPI stained female tissue

Descriptions of micrographs by number: (1) Uninucleate cells (arrow) of the apex and multinucleate progeny of these cells; multinucleate hyphal filament cells (double arrows) are at the bottom the micrograph, 120X; (2) A plate of multinucleate diaphragm cells (arrow) and associated hyphal filaments with gland cells (arrow heads), 60X; (3) A hyphal filament associated with multinucleate cells; the gland cell (arrow) is to the right and the diaphragm cell (double arrows) is to the left, 60X, (4) A close up of the diaphragm cell, with 15 nuclei, and the gland cell, with three nuclei; the attachment points for these cells to the hyphal filament are evident, 180X.





DISCUSSION

The histological results from my investigation support the findings of Bigelow (1887). Morphology of *C. parvula* is established very close to the apex. Diaphragms formed in cultured *C. parvula* within 40 μm and development of gland cells from hyphal filament cells occurred within 55 μm of the apex. Examination of sectioned and whole mount tissue did not refute Bigelow's observation that hyphal filament cells seem to give rise to diaphragm cells. For example, the ends of hyphal filament cells were always found to be above and below the diaphragm through which the cells were passing, and between cortical and diaphragm cells in histological sections. Bigelow noted that 11 - 15 hyphal filaments radiate from the apex in a branch tip and that this range of numbers typically corresponds to the number of apical cells (3 to 4) plus the number of cells that compose the two tiers of cells that crowd in around the apical cells. In the words of Bigelow (1887), each of these cells is "morphologically at the tip" of each hyphal filament. Study of serial sections revealed that hyphal filaments did originate from the apex and that there were about 11 hyphal filament cells in a branch tip, however, this number was not compared to the number of apical cells.

Although my investigation backs this early work, the developmental event that is altered or stopped in synthetic media lacking bromide remains unidentified. Sections and whole mounts of *C. parvula* tissue cultured in the synthetic treatments, particularly Sm-Br and St-Br, even over shortened time intervals (*i.e.*, making observations on Day 1 versus waiting until Day 3), did not yield clues about the formation of diaphragms. The determination of the order in which certain cell types form in *C. parvula* proved difficult in part due to the small size of cells, particularly apical cells.

The culturing experiments of Thursby and Harlin (1984) and the hypothesis that bromide is required for diaphragm formation were supported by the results from the early stages of my investigation. In two nutrient enriched synthetic seawater media (Sm and St), for both tetrasporophyte and female isolates, diaphragm formation did stop in *C. parvula* when bromide was unavailable. In nutrient enriched, synthetic media lacking bromide, first segment lengths (FSLs) are significantly large after 3 or 5 days of culture compared to: (1) controls (NSW, Sm, and St), (2) treatments lacking other individual trace metals (*i.e.*, Al, I, Li, Rb, and Sr), and (3) treatments with 25 μM or higher concentrations of bromide. With an addition of 25 μM bromide, diaphragm formation is maintained. However, at concentrations higher than 50 μM bromide diaphragm formation is less variable. Morphological features that were not affected in synthetic medium lacking bromide include: (1) the development of hyphal filaments and gland cells, (2) apical growth and the development of branches, and (3) cell expansion.

However, lengthening of the first segment eventually occurs by Day 10 or Day 14 in complete synthetic media (Sm and St) in spite of efforts to improve results in these treatments by decreasing the intervals between media changes, by varying light levels, and by altering sources of iron. Thus, the nutrient enriched synthetic media, though used as controls in the experiments, were less than optimal for maintaining diaphragm formation. Salinity and pH did not vary significantly among treatments, and therefore are not correlated with treatment effects.

That synthetic media with or without bromide does not support diaphragm formation for an extended time in culture indicates that diaphragm formation may be tied to environmental stress response in *C. parvula*. That reproductive events are not as

successful in Sm as in NSW may support the possibility that Sm and Sm-Br promote a stress in *C. parvula*. Fertilization, cystocarp production and carpospore production all take place in Sm, but not as quickly or as productively as in NSW. Fertilization is completely inhibited in Sm-Br.

Branch initiation and consequently, apical growth and cell expansion continue in both synthetic media with and without bromide. In St-Br, initiation of branches continues; however, like the main branch, the new branches lack diaphragm formation. Additionally, apical growth in St-Br is less than the growth seen in complete St and NSW. Evidence from the investigation does indicate that although diaphragms may be structurally important for maintaining the overall appearance of *C. parvula*, diaphragms may be expendable morphological features. Diaphragms do not serve as tensile or positional cues for branch initiation or for the formation of new diaphragms. The loss of diaphragm formation in synthetic medium minus bromide is a reversible event. After three days of culture in NSW, St, and St-Br, first segment length, but not tip length in the minus bromide treatment, is significantly different from first segment lengths in tissue cultured in NSW and St. When tissue is then transferred to NSW, diaphragm formation resumes, such that first segment length is not significantly different from that in the tissue originally cultured in NSW and St, by Day 6.

The initial observation that bromide was correlated with diaphragm formation was made by Thursby and Harlin (1984) during their work to formulate a synthetic medium for culture of *C. parvula*. In this study, the loss of diaphragm formation in *C. parvula* tissue was stated to have occurred in synthetic media that lacked bromide. Additionally, in this investigation, loss of diaphragm formation was seen both in St and St-Br in which

nitrate was replaced with nitrite. In contrast, Thursby and Harlin (1984) subjected female *C. parvula* to numerous stressful conditions in their investigation where loss of diaphragm formation might have occurred but did not. These conditions were as follows: (1) *C. parvula* was exposed to ten organic toxins resulting in inhibited reproduction between males and females, (2) under crowded conditions in culture, tissue turned yellow, (3) lack of vitamins inhibited growth, but growth was stimulated when EDTA was added in the absence of vitamins, (4) iron deficiency resulted in limited growth and pink tissue, (5) phosphorous deficiency resulted in white precipitate forming on tissue; this was considered to be CaCO_3 , and (6) in the absence of nitrate, tissue died, and under nitrate deficiency, tissue turned yellow. Excess branching in *C. parvula* may be related to changes in nutrient conditions during culturing (a possible source for stress) (Steele and Thursby 1980). For example, when tetrasporophytes switched from ammonia to nitrate as a nitrogen source, excess branching occurred. However, during this present investigation, excess branching was not seen in any of the treatments for female gametophytes or tetrasporophytes.

There are two accounts of lack of diaphragm formation in *C. parvula* that are unrelated to bromide availability. In an early report, Steele and Thursby (1980) stated that under their culture conditions, four female mutants resulted that exhibited a lack of diaphragm formation (Steele and Thursby 1980). Steele and Thursby (1980) speculated that diaphragm formation had either failed to form at all or completely during development. The mutant females were similar in size to normal females, and, most interestingly, fertilization occurred resulting in the appearance of normal looking cystocarps on these females. Lack of diaphragm formation has also been noted for *C.*

parvula that has been exposed to antibiotics (Alexander O. Frost, personal communication). Antibiotics work by blocking bacterial metabolism; red algal metabolism may be similarly susceptible to the effects of antibiotics. It is important to note that a close relative of *C. parvula*, *Lomentaria baileyana* (also in the Rhodymeniales, Rhodophyta) also has hollow branch tips spanned longitudinally by hyphal filaments, however, the filaments are highly branched perhaps to compensate structurally for the lack of diaphragms in this alga.

The work initiated with DAPI and the aceto-iron-haemotoxylin-chloral hydrate stains provides the most promise for determining the developmental events that occur in synthetic media with and without bromide. These stains on tissue squashes reveal information on how cells are arranged and associated in a thallus. Tissues like diaphragms and hyphal filaments fall into the category of secondary tissues in macroalgae. There is a need for more research on secondary, non-reproductive tissue in macroalgae. Much of the recent work on algal development using modern techniques has been done on primary tissues, especially the growth of shoots and rhizoids (Coomans and Hommersand 1990, Appendix C).

Future investigations into the role of bromide in *C. parvula* should perhaps rely on the use of DAPI stain with tissue squashes. The finding that the cuticle of tissue cultured in synthetic media minus bromide is not maintained and seems susceptible to microbial attack, may point to discovering a role of bromide in *C. parvula*. The discovery using DAPI that cells of *C. parvula* eventually become multinucleate is supported by the extensive red algal work performed by Goff and Coleman (1986, 1987), and provides a new line of research with this alga.

That bromide has been identified as a possible player in maintaining the morphology of a red alga is interesting given the volume of work that has been done and is being done on halogens in red algae (Butler and Walter 1993; Appendix D). New questions that have arisen from this investigation are: what are the metabolic connections between bromide availability and diaphragm formation and vegetative and reproductive health in *C. parvula*? Clues to the answer of this questions may lie in (1) figuring out the metabolic role of gland cells and hyphal filaments and determining why are they unaffected in bromide deficient media, (2) further study of the cuticle and whether or not bromide required for inhibiting microbial attack of the cuticle, and (3) in thoroughly investigating the stress responses in *C. parvula*. These lines of investigation provide direction for future studies. Perhaps, the starting point for these studies should be developing a nutrient enriched synthetic seawater medium that will support *C. parvula* morphology indefinitely.

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APPENDIX A: Recipes for media

I. Nutrient enrichment solution for natural seawater (NSW):

referred to as modified 'f' medium or GP2. Thursby and Steele (1986) developed the medium for *C. parvula* based on Guillard and Ryther (1962) and Spotte *et al.* (1984). Apple and Harlin (1995) most recently used the medium for culturing *C. parvula*.

A. Add the following compounds and solutions in order to 1 L of R-O DiH₂O:

6.35 g sodium nitrate (NaNO₃)

0.64 g sodium phosphate (NaH₂PO₄ · H₂O)

133 mg Na₂EDTA · 2H₂O)

51 mg sodium citrate (C₆H₅Na₃O₇ · 2H₂O)

19.5 ml iron solution*

10 ml vitamin solution**

* React 9.75 mg of iron filings with 2 ml of concentrated HCl for 20 minutes then bring the volume of the solution to 19.5 ml with R-O DiH₂O.

** Combine 2.0 g thiamine-HCl, 1.0 mg biotin, 1.0 mg B12 in 100 ml of de-ionized water. Filter sterilize the vitamin solution and store in 10 ml aliquots in test tubes at -20° C.

B. Autoclave the modified 'f' medium (GP2) for 10 minutes, cool and then store at 4 C.

II. Recipe for nutrient enriched synthetic seawater (Sm) based on McLachlan (1973): the procedures for this medium were developed by A.O. Frost (personal communication) and based on McLachlan (1973) for the nutrient enriched synthetic seawater and on Provasoli (1963) for the S-3 vitamin solution

A. Recipe:

1 tube of S-1 Salts (NaCl and KCl)

10 ml 2 M $MgCl_2$ *

10 ml 2 M $MgSO_4$

10 ml 1 M $CaCl_2$

5 ml SSM-T3 Macronutrients

2 ml STM-1 Trace Metals

4 ml STM-2 Trace Metals

4 ml 62.5 mM H_3BO_3 (3.86 g / L R-O DiH_2O)

4 ml S-3 Vitamins

Bring volume to 1000 ml with R-O DiH_2O .

* NOTE: When a solution of $MgCl_2$ was unavailable, 15 ml of 2 M $MgSO_4$ was added per liter to replace added 10 ml 2 M $MgCl_2$. In other words, 25 ml of 2 M $MgSO_4$ was added under this circumstance. Adding this amount of $MgSO_4$ ensured that the salinity of Sm would be at least 28 ppt.

B. S-1 Salts added directly to medium:

<u>Salts</u>	<u>Amount / L Sm</u>	<u>Final Concentration in Sm</u>
NaCl	23.36 g	400 mM
KCl	0.75 g	10 mM

* NOTE: Dry aliquots can be stored in screw cap test tubes.

C. S-2 Salt working stock solution: 10 ml / L Sm

<u>Salts</u>	<u>Amount / L Sm</u>	<u>Final Concentration in Sm</u>
CaCl ₂	0.34 g	10 mM
MgCl ₂	1.91 g	20 mM
MgSO ₄	2.41 g	20 mM

Stock solutions:

1 M CaCl₂: Add 80.07 g CaCO₃ to 135 ml concentrated HCl. Allow the mixture to react for 2 hours on a warm stir plate. Increase the volume to 800 ml with DiH₂O and store at 25 C.

2 M MgCl₂-6H₂O: Add 203.3 g to 500 ml.

2 M MgSO₄: Add 240 g of MgSO₄ to 1 L Di-H₂O.

D. SSM-T3 Macronutrients working stock solution: 5 ml / L Sm

<u>Compounds</u>	<u>Amount added to 400 ml DiH₂O</u>	<u>Amount / L Sm</u>	<u>Final Concentration in Sm</u>
NaHCO ₃	13.44 g	0.17 g	2 mM
NaNO ₃	10.2 g	0.13 g	1.5 mM
NaH ₂ PO ₄ -H ₂ O	0.8832 g	11 mg	0.08 mM
TRIS-HCl	37.83 g	0.47 g	3 mM

* NOTE: Store working stock solution (400 ml) at 4° C.

E. STM-1 Trace Metals working stock solution: 2 ml / L Sm

<u>Compounds</u>	<u>Amount per L R-O Di-H₂O</u>	<u>Amount / L Sm</u>	<u>Final Concentration in Sm</u>
MnCl ₂ -4H ₂ O	0.99 g	2 mg	10 uM
ZnCl ₂	0.68 g	1.4 mg	10 uM
CoCl ₂ -6H ₂ O	0.012 g	24 ug	0.1 uM
CuSO ₄ -5H ₂ O	0.003 g	5 ug	0.02 uM
Na ₂ MoO ₄ -2H ₂ O	0.06 g	120 ug	0.5 uM
FeC ₆ H ₅ O ₇ -5H ₂ O	0.335 g	670 ug	2 uM

A 0.2 M EDTA stock solution is used as a chelate in the salt solutions. The EDTA stock is stable at 4° C. Stock trace metals solutions are further stabilized by increasing the pH to 8 - 9. STM-1 Trace Metals working stock solution is made by combining 50 ml of each of stock salt solutions (see below for procedures) and then bringing the volume to 1 L with R-O Di-H₂O.

Procedures for making stock solutions for each trace metal. In each case, after the trace metal is added to the EDTA solution, the stock solution is adjusted to pH 8 - 9 and

R-O Di-H₂O is added to bring the final volume to 200 ml. In some cases, the original volume is lost due to heating.

Trace metals stock solutions:

Manganese: Add 3.96 g MnCl₂·4H₂O to 200 ml boiling 0.2 M EDTA in a 400 ml beaker on a stirring hot plate. Adjust pH to 8 - 9. Pour adjusted solution into a 200 ml volumetric flask and compensate for lost volume by adding R-O Di-H₂O from two rinses of the 400 ml beaker.

Zinc: Add 2.73 g ZnCl₂ to 200 ml boiling 0.2 M EDTA . Follow the procedure as above.

Cobalt: Add 0.0476 g CoCl₂·6H₂O to a boiling solution of 2 ml 0.2 M EDTA and 100 ml R-O Di-H₂O. Follow procedure as above.

Copper: Add 0.01 g CuSO₄·5H₂O to a boiling solution of 0.4 ml 0.2 M EDTA and 100 ml R-O Di-H₂O. Follow the procedure as above. For a sulfate-free solution, substitute CuCl₂).

Molybdenum: Add 0.24 g Na₂MoO₄·2H₂O to a boiling solution of 10 ml 0.2 M EDTA and 100 ml R-O Di-H₂O. Follow the procedure as above.

Iron: Add 1.34 g FeC₆H₅O₇·5H₂O to a boiling solution of 40 ml 0.2 M EDTA and 100 ml R-O Di-H₂O. Follow procedure as above.

F. STM-2 Trace Metals working stock solution: 4 ml / L Sm

	Amount per 200 ml		Final Concentration in Sm
	<u>R-O Di-H₂O</u>	<u>Amount per liter Sm</u>	
NaBr	30.87 g	31 mg	301 uM
SrCl ₂ -6H ₂ O	16.00 g	16 mg	60 uM
AlCl ₃ -6H ₂ O	0.12 g	120 ug	0.5 uM
RbCl	0.24 g	240 ug	2 uM
LiCl	0.42 g	420 ug	10 uM
NaI	0.02 g	20 ug	0.13 uM

* NOTE: Each 200 ml trace metal stock solution is alkalated with 1 N NaOH and stored at 4 C. The STM-2 working stock is made by adding 10 ml of each of the trace metal solutions and bringing the final volume to 200 ml with R-O Di-H₂O.

G. S-3 Vitamins:

	Amount per 400 ml	
	<u>R-O Di-H₂O</u>	<u>Amount / L Sm</u>
i-Inositol	20 g	2 mg
Thymine	12 g	1.2 mg
Thiamine-HCl	2 g	200 ug
Nicotinic acid	0.4 g	40 ug
PABA	0.04 g	4 ug
Folic acid	0.008 g	0.8 ug
Biotin	0.004 g	0.4 ug
Cyanocobalamin (B ₁₂)	0.004 g	0.4 ug
Ca- panthothenate	0.4 g	40 ug

Preparation of 400 ml working stock solution of S-3 vitamins:

Add 20 g of i-Inositol to 300 ml R-O Di-H₂O in a 500 ml beaker on a stirring hot plate. Acidify to near pH 4.5 with concentrated HCl. Add other vitamins, except Ca-pantothenate in the order listed above. The solution should be gently heated and stirred during these additions. Adjust the pH to 4.5 if necessary and autoclave the solution.

Dissolve 0.5 g of Ca-pantothenate in 25 ml Di-H₂O at room temperature.

After autoclaved solution is cool, add 20 ml of the Ca-pantothenate solution via sterile filtration. Bring this solution up to 400 ml with sterile R-O Di-H₂O and add to sterile storage bottles. Store at - 70 C.

Preparation of working stock solution of S-3 vitamins:

Thaw S-3 stock bottle and remove 1 ml under sterile conditions. Add the 1 ml of S-3 via sterile filtration to 100 ml of sterile R-O Di-H₂O. Aseptically dispense 2 ml aliquots in to sterile 10 ml screw top test tubes. Store aliquots at -20 C.

H. Special conditions:

- * Sulfate-free media: Omit MgSO₄ and replace volume with MgCl₂ (adding a total volume of MgCl₂ of 20 ml). Additionally, replace STM-1 with STM-1 minus -SO₄.
- * When 2M MgCl₂ was unavailable, it was replaced with 1.5x volume of 2M MgSO₄.

III. Recipe for St, nutrient enriched synthetic seawater used by Thursby and Harlin (1984):

A. General Purpose Medium 2 (GP2)

	<u>Amount / L St</u>	<u>Final Concentration in St</u>
NaCl	21.03 g	360 mM
Na ₂ SO ₄	3.52 g	25 mM
KCl	0.61 g	8 mM
KBr	0.088 g	739 mM
Na ₂ B ₄ O ₇ -10H ₂ O	0.034 g	89 mM
MgCl ₂ -6H ₂ O	9.5 g	50 mM
or MgCl ₂	4.45 g	
or 4.9 M MgCl ₂ -6H ₂ O	10.2 ml	
CaCl ₂ -2H ₂ O	1.32 g	9 mM
or CaCl ₂	0.997 g	
or 2 M CaCl ₂	4.5 ml	
SrCl ₂ -6H ₂ O	0.02 g	75 uM
or 300 mM solution	0.25 ml	
NaHCO ₃	0.17 g	2 mM
or 400 mM solution	5 ml	
Nutrient media	5 ml	

* NOTE: Add these ingredients to 1 L of R-O Di-H₂O to make 1 L of St. The 4.9 M MgCl₂-H₂O solution (1 g MgCl₂-H₂O per ml) was obtained from Sigma, St. Louis, MO.

B. Nutrient media: Working stock solution (5 ml / L St)

	<u>Stock solution</u> <u>Amount / L</u>	<u>Amount / L St</u>	<u>Final Concentration</u> <u>in St</u>
NaNO ₃	12.7 g	127 mg	1.5 mM
NaH ₂ PO ₄ -H ₂ O	1.28 g	12.8 mg	93 uM
Na ₂ EDTA-2H ₂ O	266 mg	2.66 mg	7 uM
Na ₃ C ₆ H ₅ O ₇	103 mg	1.03 mg	0.4 uM
Iron**	19.5 mg	195 ug	
Trace Elements:			
Na ₂ MoO ₄ -2H ₂ O	2.42 mg	24.2 ug	0.1 uM
KI	8.3 mg	83 ug	0.5 uM
ZnSO ₄ -7H ₂ O	2.18 mg	21.8 ug	0.076 uM
NaVO ₄	0.92 mg	9.2 ug	0.05 uM
MnCl ₂ -4H ₂ O	140 mg	1.4 mg	7 uM
MnSO ₄ -H ₂ O	61 ug	0.61 ug	
Vitamins:			
	10 ml aliquot:		
Thiamine-HCl	2 mg	2 mg	
Biotin	100 ug	1.0 ug	
B12	100 ug	1.0 ug	

* NOTE: For the working stock solution, add the amounts of ingredients listed in the first column to 1 L of R-O DiH₂O.

** NOTE: Iron and a vitamin solution added as a 10 ml aliquot is added in the same manner listed for the GP2 medium added to NSW (see above, IB.).

IV. Additional solution:

FAA (formalin-acetic acid)

50 ml 95% Ethanol

5 ml Glacial Acetic Acid

10 ml 37% Formalin

35 ml DiH₂O

Compound	Amount (g / L)	
	3m	50
	25.36	0.01
KCl	9.7456	0.01
MgCl ₂	1.904	0.01
NaClO ₂	1.408	—
CaCl ₂	3.12	0.001
Na ₂ SO ₄	—	0.50
K ₂ Cr ₂ O ₇	—	0.001
Ca ₃ (PO ₄) ₂ · 10H ₂ O	—	0.014
Ca ₃ (PO ₄) ₂	0.016	0.02
NaHCO ₃	0.158	0.17
Na ₂ WO ₄	0.191	0.127
Na ₂ HPO ₄ · 12H ₂ O	0.0174	0.0125
Na ₂ HPO ₄ · 12H ₂ O	0.0112	0.0066
Zn ₂ (C ₂ H ₃ O ₂) ₂	0.01045	0.00101
Iron	0.00076	0.00005
FeCl ₃ · 6H ₂ O	0.0021	—

V. Direct comparison of nutrient enriched synthetic seawater media, Sm and St:

Type of Nutrient	Name of Compound	Amount (g / L)	
		Sm	St
Salts	NaCl	23.36	21.03
	KCl	0.7456	0.61
	MgCl ₂	1.904	4.45
	MgSO ₄	2.408	---
	CaCl ₂	2.22	0.997
	Na ₂ SO ₄	---	3.52
	KBr	---	0.088
	Na ₂ B ₄ O ₇ ·10H ₂ O	---	0.034
	SrCl ₂ ·6H ₂ O	0.016	0.02
	NaHCO ₃	0.168	0.17
Macro-nutrients	NaNO ₃	0.191	0.127
	NaH ₂ PO ₄ ·H ₂ O	0.0174	0.0128
	Na ₂ EDTA·2H ₂ O	0.00133	0.00266
	Na ₃ C ₆ H ₅ O ₇	0.000448	0.00103
	Iron	0.000098	0.000195
	TRIS-HCl	0.4728	---

Type of Nutrient	Name of Compound	Amount (g / L)	
		Sm	St
Trace Elements	ZnSO ₄ -7H ₂ O	---	0.000022
	H ₃ BO ₃	0.01546	---
	KI	---	0.000083
	NaMoO ₄ -2H ₂ O	0.000121	0.0000242
	LiCl	0.000424	---
	RbCl	0.000242	---
	NaVO ₄	---	0.0000092
	MnCl ₂ -4H ₂ O	0.00198	0.0014
	ZnCl ₂	0.001363	---
	FeC ₆ H ₅ O ₇ -5H ₂ O	0.00067	---
	CoCl ₂ -6H ₂ O	0.000024	---
	CuSO ₄ -5H ₂ O	0.000005	---
	NaBr	0.03087	---
	AlCl ₃ -6H ₂ O	0.000121	---
	NaI	0.000022	---
	C ₆ H ₅ Na ₃ O ₇ -2H ₂ O	0.00051	---

Type of Nutrient	Name of Compound	Amount (g / L)	
		Sm	St
Vitamins *Amounts are in mg/L	Thiamine-HCl	200.5	1.95
	Biotin	0.101	0.001
	B ₁₂	0.101	0.001
	i-Inositol	5.0	---
	Thymine	3.0	---
	Nicotinic Acid	0.1	---
	PABA	0.01	---
	Folic acid	0.002	---
	Ca-Panthenate	0.1	---

APPENDIX B:

Table 1A

Details of described experiments plus values for Day 0 listed according to corresponding figure numbers. Descriptions and corresponding notation for treatment media are provided in Table 1 in the text. Abbreviations: T = tetrasporophyte; F = female; ** = Figure 10 represents a preliminary experiment that involved extensive morphometric measurements of *C. parvula* tetrasporophyte branch tips (see Materials and Methods, under Experimental culturing conditions).

Figure	Treatment	Day 0	Day 1	Day 2	Day 3
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Table 1A

Fig	Tis- sue type	Treatment Media	n	Day(s) changed	Light level μmol $\text{m}^{-2} \text{s}^{-1}$	Day 0 FSL \pm 2S.E. (mm); n value
2a, 2b 3	T	Sm; Sm-TM; Sm-Br, -Al, -I, -Li, -Rb, & -Sr; and NSW (in 2 of 4 runs)	10	7	30-40	0.12 ± 0.014 ; 40
5a, 5b	T	NSW; Sm; Sm w/ 0, 25, 50, 100, 150, & 200 μM bromide	15	5	30-40	0.10 ± 0.01 ; 60
6a, 6b	T F	NSW; St; St-Br	a, 10 b, 10	3	30-40	T: 0.12 ± 0.06 ; 20 F: 0.09 ± 0.026 ; 20 T&F: 0.11 ± 0.02 ; 40
7	T F	NSW; St; St-Br	30	5, 10	14-22	T: 0.93 ± 0.14 ; 10 F: 0.07 ± 0.02 ; 10 T&F: 0.5 ± 0.2 ; 20
8	T F	NSW; StFC; StFS	30	5, 10	14-22	T: 0.08 ± 0.014 ; 10 F: 0.07 ± 0.024 ; 10 T&F: $0.075 \pm$ 0.014 ; 20
9a - c	F	NSW; St; St-Br; St+NO ₂ ; St-Br+NO ₂	5	no change	30-40	0.09 ± 0.02 ; 10
10 **	T	NSW; Sm; Sm- TM	18	8	30-40	0.13 ± 0.022 ; 6

APPENDIX C: Development in red algae

In discussing the development of red algae, Waaland (1990) lists events that typically must occur during development. Cell number increases, cell size and shape changes, and cells differentiate. As a function of the apical regions of branch tissue, continuous development multicellular diaphragms in *C. parvula* would require repeated cell division, variation in the plane of cell division, cell expansion, and designation of cell function. Additionally, the timing of these developmental events would be important.

There is good documentation of the patterns of development for red macroalgae (Boney and Corner 1962, Dixon 1971, Dixon 1973, Coomans and Hommersand 1990). However, investigations of the factors which induce or affect the fundamental developmental processes such as cell division, expansion, and differentiation are few in number (Dixon 1971, Dixon 1973, Murray and Dixon 1975, Waaland 1984, Waaland 1990). The environmental morphogenic factors that influence algal macrophytes include levels and types of irradiation, nutrition, gravity, herbivory, and surface energy (Lobban and Harrison 1994). Regarding the red algal literature, irradiation is perhaps the most investigated of these factors (Dixon 1973, Waaland 1990, Murray and Dixon 1992). For example, photoperiod (Edwards 1977, Murray and Dixon 1975, Garbary 1979) and light intensity (Boney and Corner 1962, Edwards 1977) can affect the rate of cell division. And, photoperiod (Waaland and Cleland 1972, Garbary 1979, Sylvester 1987 xx) and unidirectional light (L'Hardy-Halos 1971, Waaland *et al.* 1977, Neushul *et al.* 1967) have been implicated in the regulation of cell expansion. Other factors examined in red algal morphogenesis include the age of a cell (Murray and Dixon 1975, Garbary 1979), cell position in the thallus (Duffield *et al.* 1972, Murray and Dixon 1975), and cell tissue

type, either rhizoidal or of the shoot (Neushul *et al.* 1967, Waaland and Cleland 1972, Waaland *et al.* 1977).

The differentiation of primary tissue such as rhizoidal and shoot tissue in algal macrophytes is relatively well studied (Quatrano 1990, Waaland 1990, Berger *et al.* 1994). Red algae are particularly useful subjects for studies on cell differentiation because cellular events occur within the confines of simple construction and because vegetative regeneration and high growth rates facilitate their culture (Dixon 1971, Waaland 1990). For example, excised tissue from many red algae will regenerate by developing shoot cells from the end of the filament or cell that was nearest the apex and rhizoidal cells from the end that was nearest the base of the thallus (Dixon 1973, Waaland and Cleland 1974). Similar phenomena have not been explored for the development of secondary tissue (*i.e.*, the hyphal and diaphragm tissue in *C. parvula*) in red algae (Coomans and Hommersand 1990). Physical factors that affect cell differentiation in primary red algal tissue include cell position in the thallus (L'Hardy-Halos 1971, Duffield *et al.* 1972) and proximity to a site of wounding (Duffield *et al.* 1972, Waaland 1986, personal observation). To note, recent work in the area of red algal molecular biology has determined that red algae do not have true cell differentiation as defined by that seen in higher plants and animals (Stiller and Waaland 1996, Stiller and Hall 1998).

In light of what is known about the activity of hormones in higher plants, there is a reasonable amount of speculation on hormones in algal macrophytes (Dixon 1973, Murray and Dixon 1975, Lobban and Harrison 1994). Produced at the cellular level, hormones, also known as growth substances, are considered to be endogenous morphogenic factors (Waaland 1990, Murray and Dixon 1992, Lobban and Harrison

1994). To date, the only identified and characterized endogenous growth substance for macroalgae is rhodomorphin (Waaland 1975, Waaland 1984, Watson and Waaland 1986). Rhodomorphin, found only in red algae, is produced by repair cells which elongate in a rhizoidal manner to bridge a gap of dead tissue created by wounding (Waaland and Waaland 1975, Waaland 1984, Watson and Waaland 1986). As a wound response substance, rhodomorphin appears to induce cell division and differentiation, and cell-cell attraction during repair events (Waaland and Waaland 1975, Waaland 1984, Watson and Waaland 1986). The activity of isolated rhodomorphins is species-specific (Waaland and Waaland 1975, Waaland 1984, Watson and Waaland 1986). In a more recent study, a rhodomorphin-like substance was localized in a red alga using fluorescently-labeled lectins; like rhodomorphin, the compound induces cell attraction and polar growth during repair and is a glycoprotein (Kim and Fritz 1993). Aside from the work of Waaland (Waaland and Waaland 1975, Waaland 1984, Watson and Waaland 1986) and Kim & Fritz (1993), research on isolated compounds or the effects of known growth hormones (*i.e.*, auxin) has not yielded clear evidence for the presence of endogenous morphogenic chemicals in any algal macrophyte (Waaland 1984, Lobban and Harrison 1994).

APPENDIX D: Halogens in Algae

An attempt was made in this study to detect peroxidase activity in *C. parvula*.

The rationale for this search was based on the following literature review.

Halides and halogenated compounds in macroalgae

Synthetic seawater media formulations are mixtures of “plant nutrients”: salts, nitrogen, phosphorus, calcium, trace metals, and vitamins, with buffers and chelators added as stabilizers (McLachlan 1973). The halides, iodide and bromide, are commonly included among the added trace metals to synthetic media (McLachlan 1973) and readily available in natural seawater with a salinity of 35 ppt. The average concentrations for bromide and iodide at this salinity are 65 mg/L (814.3 μM) for Br⁻ and 0.06 mg/L (0.4 μM) for I⁻ and IO³⁻, respectively (Goldberg 1963).

Macroalgae (and other marine organisms) have easy access to halogens in the marine environment and, not surprisingly, halogenated compounds are commonly found in many marine organisms (Butler and Walker 1993). Among the red algae, representatives from nine families have been found to contain halogenated secondary metabolites (Fries 1966, Fenical 1975, Fries 1975, Wolk 1968). Bromine in the form of bromide appears to be the most commonly incorporated (Fenical 1975). There is some speculation on the metabolic roles for sequestered halides and halogenated compounds. The putative role of halides and halogenated compounds in macroalgae is that they act as protective compounds against bacteria, epiphytes or herbivores (Fenical 1975, Butler and Walker 1993, Laturus *et al.* 1996). Haloperoxidases, required for sequestering halogens and synthesizing halogenated compounds, may mediate adhesion in algae by cross-

linking polymeric or polyphenolic substances (Johnson *et al.* 1995, Vreeland *et al.* 1998).

Studies that have addressed the requirement of halides for development and growth in macroalgae are few. At least one study found that the refractile inclusions in a certain red algal genera (family Bonnemaisoniaceae) seemed to be a storage site for bromide (Wolk 1968). When bromide was excluded from a synthetic culture medium, the refractile vesicles failed to form (Wolk 1968). Fries (1966 and 1975) discovered that *Polysiphonia* has an absolute demand for iodide and that optimal growth requires both iodide and bromide. The zoospores of the brown alga, *Petalonia*, were found to follow different developmental pathways according to the availability of iodine (Hsiao 1969). McLachlan (1977) found that normal growth of *Fucus* embryos required at least 50 μM bromine. Interestingly, bromine was implicated as a requirement in *Fucus* after the finding that omission of a trace metal solution containing bromine greatly inhibited growth (McLachlan 1977). Most recently, stalk formation and attachment in a marine diatom were found to be bromide-dependent (Johnson *et al.* 1995). However, high concentrations of iodide inhibited both stalk formation and diatom attachment to a substratum, even in the presence of bromide (Johnson *et al.* 1995). However, stalk formation was also inhibited despite bromide availability when the diatoms were grown in sulfate-free media (Johnson *et al.* 1995).

Haloperoxidases in macroalgae

Johnson *et al.* (1995) proposed that bromide acts as a substrate for an endogenous peroxidase. By crosslinking the extracellular polysaccharides secreted by the marine diatom, such a bromide-dependent peroxidase could render the compounds insoluble and appropriate for stalk formation (Johnson *et al.* 1995, Vreeland *et al.* 1998). These findings are supported by the work of Vreeland *et al.* (1998). Vanadate bromoperoxidase has been identified in *Fucus* embryos by Vreeland *et al.* (Vreeland *et al.* 1994 and 1995). The activity of vanadium- and bromide- dependent peroxidase (Vreeland *et al.* 1994, Vreeland *et al.* 1995) and the attachment of *Fucus* zygotes (Crayton *et al.* 1974) requires sulfate. Attachment is thought to be mediated, at least in part, by the peroxidase which indirectly activates secreted polyphenols to “crosslink wall polymers to form a glue which binds to both hydrophobic and hydrophilic surfaces” (Vreeland *et al.* 1995). More recently, Vreeland *et al.* (1998) has proposed a “fiber-phenolic-catalyst mechanism of alga adhesion” within the cell wall matrix. Polyphenolics are activated to bond non-specifically to acidic carbohydrate fibers and to a substratum via the catalytic activity of vanadium bromoperoxidase. The complete details of the mechanism are not fully understood due to the difficulties in working algal peroxidases explained in detail in Vreeland *et al.* (1998) and discussed in personal communication with Dr. V. Vreeland (1996).

The history of identifying haloperoxidases in macroalgae are as follows. Murphy and Ó hEocha (1973) provided the first respected evidence for the presence of peroxidase activity in red algae; research from 30 years before had identified peroxidase but the conclusions were tainted by claims that the results were based on artifacts. The interest in

isolating peroxidase from a red alga was inspired by the possibility that peroxidase mediates the biosynthesis of halogenated compounds (Murphy and Ó hEocha 1973). Upon isolating peroxidase, Murphy and Ó hEocha (1973) found that their compound was unable to oxidize iodide, tested because it has the lowest redox potential for $2X^- \rightarrow X_2$ where X represents a halide. The researchers concluded that the isolate was not involved in the synthesis of halogenated compounds; they did not test to see if the peroxidase reacted with bromide (Murphy and Ó hEocha 1973). However, Pedersén (1976) later characterized a brominating peroxidase from this alga. Hewson & Hager (1980) found that 55 out of 72 marine algae tested had bromoperoxidase activity, and that the majority of the 55 were representatives of Rhodophyta. A vanadium-dependent bromoperoxidase has been recently found in *Corallina officinalis*, a red alga (Sheffield *et al.* 1993). Vreeland *et al.* (1996) and Waite *et al.* (1997) localized vanadium-dependent bromoperoxidases in attached *Ectocarpus* spores and in the mucilage of *Fucus* embryos (both brown algae) and in the frustule of the diatom, *Achnanthes*.

Conclusion

Haloperoxidases may be involved in cell wall activity in macroalgae or simply in producing unique secondary metabolites. The peroxidase work with *C. parvula* was not conclusive and this may be in part due to not having had enough tissue from this hollow, delicate alga to detect peroxidase activity. Vreeland *et al.* (1998) offers some consolation: "The inability to detect either phenolic compounds or haloperoxidases (Moore and Okuda 1996) in algal extracts is not necessarily an indication that these compounds are not present (*e.g.* see Mehrtens 1994). Some cultured Antarctic brown

algae showed no halogenating activity in crude extracts, although they produced large amounts of polyhalomethanes, which can result only from a halogenation reaction (Laternus *et al.* 1996).” Vreeland *et al.* (1998) explain further that detection of haloperoxidases and involved polyphenols is very difficult due to molecular interactions and reactions with algal carbohydrates.

The role of halogenated compounds in algal systems is not well understood (Laternus *et al.* 1996). A push toward understanding the role within macroalgae has not occurred among scientists for four possible reasons: (1) interest in halogenated compounds has leaned toward uncovering their pharmacological uses particularly since the compounds seem to work as antimicrobial and antiviral agents (Butler and Walker 1993), (2) the concern that ozone depletion particularly over Antarctica is due to volatile halogenated compounds from macroalgae (Laternus *et al.* 1996), (3) the difficulty in working with algal haloperoxidases which are required for synthesizing halogenated compounds (Vreeland *et al.* 1998), and (4) the seemingly underdeveloped field macroalgal developmental biology outside of the investigations on *Fucus* embryos. The latter reason may in part be explained with the fact that present day understanding of animal and higher plant developmental biology is relatively much greater than the present day understanding of macroalgal developmental biology. The imbalance in understanding in this narrow field is comparatively true for many other topics in biology when one does a cross comparison of scientific effort by kingdom.

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