Hydrobiologia **398/399:** 253–259, 1999.
J.M. Kain (Jones), M.T. Brown & M. Lahaye (eds), Sixteenth International Seaweed Symposium,

Using *in situ* substratum sterilization and fluorescence microscopy in studies of microscopic stages of marine macroalgae

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Key words: fluorescence microscopy, microscopic stages, recruitment, seed banks, sterilize

Abstract

The methods currently used for examining the relative contribution of microscopic stages to the persistence of natural populations of marine macroalgae can be inappropriate for use in subtidal habitats. Also, because of their microscopic size, direct examination and obtaining an estimate of recruitment, growth and mortality of these stages in the field is difficult. A method of removing microscopic algal stages from natural rock surfaces using watertight tents and water-soluble chemicals is presented. Also discussed is the use of a previously described method of fluorescent labelling these microscopic stages that, when examined under UV light, allows for their precise identification and growth to be determined. Together, these methods can be effective in examining the ecology of algal microscopic stages in the field.

Introduction

The factors influencing recruitment and persistence of marine macroalgae have received considerable attention in the ecological literature. Mostly, these studies have focused on the macroscopic stages (see reviews in Dayton, 1985; Schiel & Foster, 1986; Santelices, 1990; Vadas et al., 1992), but recently the contribution of microscopic stages to the maintenance of natural populations has been examined (Klinger, 1984; Hoffman, 1987; Hoffman & Santelices, 1991; Santelices et al., 1995; Edwards, 1996). For some species, these microscopic stages have been referred to as 'banks of microscopic forms' (Chapman, 1986) because, much like terrestrial seed banks, they persist during periods when the macroscopic stages are absent (Santelices et al., 1995). However, in contrast to the seeds of most terrestrial plants, algal microscopic stages are typically extremely small, making it difficult to directly observe their recruitment, growth and survivorship in the field (Dayton, 1985). Although it is assumed that the microscopic stages of at least some macroalgae, especially the kelps (order Laminariales), persist as filamentous gametophytes during periods when their macroscopic stages are absent (Kain, 1975; Klinger, 1984; Dayton, 1985; Reed et al., 1997), the precise identification of these stages (spore, gametophyte or embryonic sporophyte) remains largely undescribed in the field (but see Hsiao & Druehl, 1973).

A variety of experimental methods have been used to examine the importance of microscopic stages for maintaining populations of marine macroalgae. One of the most common has been to compare recruitment of algal macroscopic stages on sterilized (− microscopic stages) and non-sterilized (+ microscopic stages) substrata, and then to attribute any differences in recruitment to the presence or absence of microscopic stages (Santelices et al., 1995; Blanchette, 1996; Edwards, 1996; Reed et al., 1997). Although sterilizing natural rock substratum has commonly been done in intertidal and subtidal habitats, the physical nature of these environments has led to the use of very different methods. A standard approach to sterilizing surfaces in intertidal habitats has been to first scrape all visible organisms and then apply chemicals such as oven cleaner, formalin, alcohol or phosphoric acid, or to burn the substratum with a propane torch (see review by Littler & Littler, 1985). These methods work quite well in intertidal habitats (Wilson, 1925; Blanchette, 1996), but they may be inappropriate in

subtidal habitats because the chemicals must be both water-soluble, so as not to leave a toxic residue, and able to remain on the substratum long enough to kill all microscopic organisms. Burning these substrata is problematic because, although some torches may function quite well underwater, they burn too hot and can potentially be damaging to the substratum.

Standard approaches to sterilizing substrata in subtidal and tide pool habitats have typically involved manipulating small pieces of broken substratum or boulders. These are collected in the field, brought back to the laboratory and sterilized for several minutes using an autoclave (Santelices et al., 1995) or for several days in the sun (Reed et al, 1997). Once sterilized, the boulders are returned to the field and placed alongside unsterilized ones and monitored for algal recruitment. Although relatively simple and effective, this may be inappropriate in areas where the natural substratum is solid bedrock and boulders are absent. In these types of habitats, the top few centimetres of the substratum can be chipped away with a hammer and chisel (Reed & Foster, 1984), but this is not recommended because exposing new rock may cause leaching of toxic chemicals (Littler & Littler, 1985). Given these difficulties, outplanting of artificial substrata to the field at specified times is often used. Recruitment of macroscopic algal stages on these substrata is then correlated with the availability of nearby reproductive adults (Kain, 1975). Although using artificial substrata may be informative in some situations, they can also be misleading because typically they lack the refuges characteristic of natural substrata. This can lead to artificially high susceptibility of the microscopic stages to grazing (Dayton, 1985). In addition, because of the specific nature of their surfaces, the artificial substrata themselves can bias which species occur on them (Harlin & Lindbergh, 1976; Flavier & Zingmark, 1993). Given these difficulties, a method that will allow for the sterilization of natural, subtidal rock substrata *in situ* together with the use of a previously described method of identifying specific microscopic stages in the field are presented.

Material and methods

Substratum sterilization

All field experiments were done in a giant kelp (*Macrocystis pyrifera*) forest located in Stillwater Cove, California, U.S.A. (36° 34′ N, 121° 56′ W). The study site was a 40 m diameter low-relief terrace, located at a depth of 12 m near the middle of the cove. The substratum consisted primarily of solid, conglomerate and granite rock and a few small boulders. Three 0.25 m^2 experimental plots with relatively flat surfaces were identified and marked at the corners by nailing yellow plastic tape to the substratum. The plots were then prepared for sterilization by removing all macroscopic (> 0.1 cm) organisms with a knife and then abrading the substratum with a wire brush. Two $\sim$5 cm deep $\times$ 0.635 cm diameter holes were drilled in the middle of two opposing sides of each plot with a pneumatic drill, and an 18 cm long $\times$ 0.635 cm diameter stainless steel threaded rod was cemented into each hole. The following day, a 0.25 m^2 sterilization tent was placed over each plot and attached to the threaded rods with stainless steel nuts. These tents were made from black plastic sheeting attached to 5 cm anglestock PVC frames (see Figure 1). A 16 cm piece of surgical tubing penetrated the middle of each tent and served as a valve which could be opened or closed by untying or tying the tubing. A $\sim$2.5 cm thick gasket made from modelling clay wrapped in thin plastic (to prevent it from dissolving) was attached to the base of each tent such that tightening the nuts on the threaded rod forced a water-tight seal between the tents and the substratum. To check for leaks in the tent-substratum interfaces, 50 ml of fluoricine dye (1 g fluoricine per 50 ml filtered seawater) was injected into each tent with a hypodermic needle and the tent perimeter examined for leaking dye. Leaks were easily identified and plugged with petroleum-base clay, making the tents watertight.

To remove all microscopic algal stages from the experimental plots, 1 l of household bleach was injected into each tent through its valve with a large syringe. The valve was then closed and the tents left for two days. No damage was observed to the plants immediately outside the tents and subsequently *Desmarestia* recruits were observed directly on the outside edges of the sterilized plots. At the time of tent removal, all macroscopic algae were scraped from three additional 0.25 m^2 plots, and three unmanipulated control plots were established by marking them at the corners with yellow tape. To examine the efficiency of the sterilization method, a second set of three sterilized and three 'scraped-only' plots were established using the methods described above, but immediately following removal of these tents, a $\sim$10 cm^2 piece of substratum was removed from each plot using a hammer and chisel. These substrata were individu-

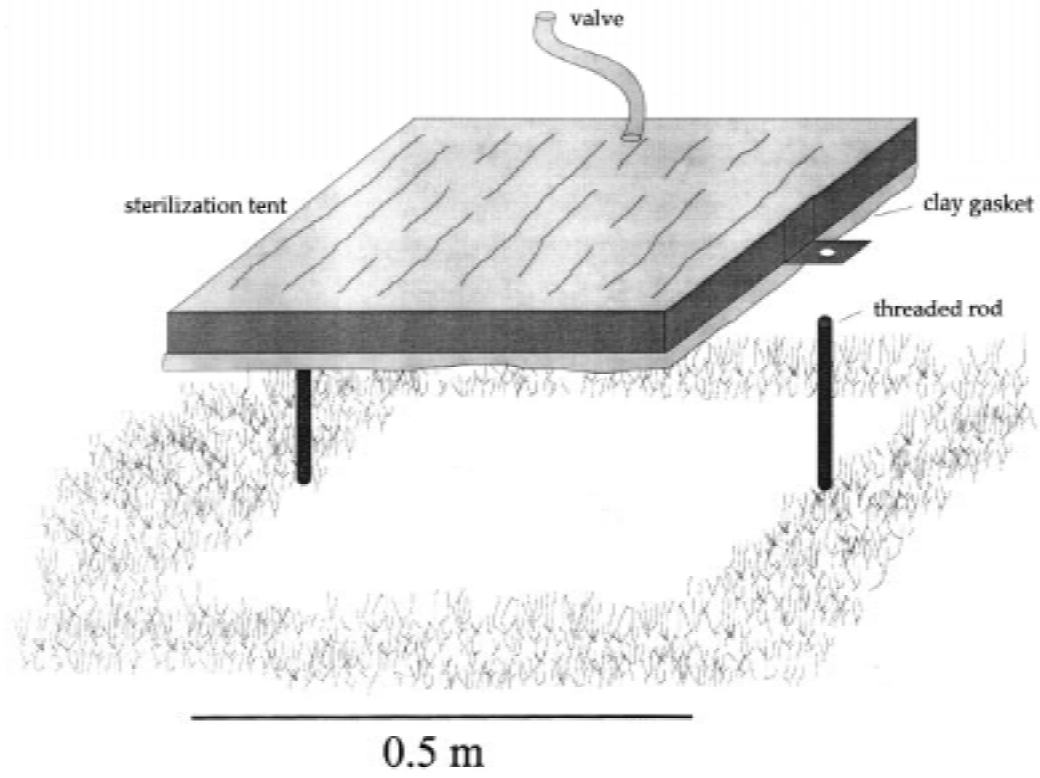

Figure 1. Diagram of sterilization tents used to remove all microscopic stages from experimental substrata. Tents measure 0.25 m^2 and were made out of reinforced plastic tarping attached to PVC frames (n = 3).

ally placed in black, plastic bags to avoid exposure to direct sunlight and transferred to the laboratory where they were placed in individual culture dishes with nutrient-enriched, 0.2 μm filtered seawater. The substrata were then incubated under an irradiance of 75 μmoles m^{-2} s^{-1}, a temperature of 9 °C and a light:dark photoperiod of 16:8 h for two months. The culture medium was changed weekly, and all substrata were monitored for the appearance of macroscopic algal stages. During this time, all experimental field plots were also monitored for recruitment of macroalgae. Monitoring of these field plots continued for two years in order to examine long-term recovery of perennial turf algae. Differences in algal densities among the field plots at both six weeks and two years after tent removal were each tested with a one-way Model I analysis of variance (ANOVA). A Bonferroni-corrected planned comparison was then used to examine differences between the sterilized and scraped-only plots six weeks after tent removal. Differences in recruitment on sterilized vs. scraped-only substrata cultured in the laboratory were examined using a t-test.

Examination of microscopic stages

To identify specific algal microscopic stages in the field, zoospores of the kelps *Macrocystis pyrifera* and *Pterygophora californica* were each settled onto glass microscope slides overnight. The following day, these slides were immersed in a 20% solution of *Fungi-Flour*™ (0.01% Cellufluor®) and filtered seawater for 24 h. *Fungi-Fluor*™ (Polysciences, Inc., Warrington, PA 18976; CAT# 17442A) is a non-lethal biostain that binds non-specifically to beta-linked polysaccharides and fluoresces (emittence 400–440 nm) when excited by UV light (240–400 nm; peak excitation 345–365 nm; Baselski & Robinson, 1989). This staining method has been used previously to stain the cell walls of laminarian (Cole, 1964; Hsiao and Druehl, 1973) and fucalean (Nakazawa et al., 1969; Serrão et al., 1996) microscopic algal stages and was found to be non-toxic and have no effects on cell growth (Nakazawa et al., 1969). After 24 h, the slides were removed from the staining solution and transferred to individual culture dishes containing nutrient enriched (70 ml Alga Grow® solution l^{-1} seawater) filtered

seawater. At this time, additional unstained zoospores of *M. pyrifera* and *P. californica* were settled onto these and other slides, yielding the following treatment combinations: stained *M. pyrifera* alone, stained *P. californica* alone, stained *M. pyrifera* + unstained *P. californica*, stained *P. californica* + unstained *M. pyrifera*, unstained *M. pyrifera* alone and unstained *P. californica* alone (n = 5 dishes per treatment). All dishes were then cultured under 40 μmoles m^{-2} s^{-1}, 15 °C, 14:10 L:D conditions for five weeks; each week, two dishes from each treatment were randomly selected and the microscopic thalli on their slides examined under UV with fluorescence microscopy (transmittance 330–385 nm).

To determine if the above method was effective at identifying the ages and growth rates of specific microscopic algal stages in the field, zoospores of the annual alga *Desmarestia ligulata* were settled onto frosted glass microscope slides overnight and stained using the methods described above. The slides were then placed on three PVC slide racks (30 slides per rack), transferred to the field in opaque, plastic bags and bolted to the substratum at a depth of 12 m. Three slides per rack were then collected each week for six weeks and examined under UV light. After the stained thalli were identified, their size (longest axis length) was measured with an ocular micrometer using standard light microscopy at 100×. Growth rates were compared between field-grown thalli and those cultured under similar conditions (75 μmoles m^{-2} s^{-1}, 9 °C, 16:8 h) in the laboratory. Although these laboratory conditions were established on the basis of measurements made in the field at the beginning of the study, average irradiance and nutrient levels were consistently higher in the laboratory.

Results

Substratum sterilization

No macroalgae recruited to any of the substratum pieces removed from sterilized plots and then cultured in the laboratory, whereas numerous (18.33 ± 9.71 recruits 10 cm^{-2}, mean ± SE) individuals, mostly fleshy red algae and unidentified Laminariales, were observed on substratum pieces removed from scraped-only plots. These differences were not significant (t-test: p = 0.34), most likely due to low statistical power (> 0.1; Cohen, 1988) which resulted from insufficient replication and high variation among the scraped substratum.

Differences in initial recruitment of macroalgae among the field plots, six weeks after tent removal, were also not significant (ANOVA: $F_{2,6}$ = 4.38; p = 0.066), although differences between sterilized and scraped-only plots were significant (Bonferroni = 0.03). No macroalgae were observed in any of the sterilized plots, while numerous individuals (15.0 ± 6.8 recruits per plot, mean ± SE), of mostly *D. ligulata*, *M. pyrifera* and *P. californica*, were observed in each of the scraped-only plots. Taken together, these results indicate that no macroalgae recruited to any sterilized substratum while significantly more macroalgae were observed on scraped-only substratum (combined probability: p = 0.04; Rice, 1990). This indicated that the method of sterilization effectively removed the algal microscopic stages while scraping the substratum alone did not.

A few weeks after tent removal, benthic diatoms were abundant on all scraped-only substrata, but no diatoms were observed on any of the sterilized substrata. Furthermore, three months after tent removal, macroalgae were found growing at all the field plots. Together, this suggested that the method of substratum sterilization did not leave a toxic residue that might prevent algal recruitment. Long-term monitoring of field plots further indicated that since there were no differences in turf algal abundance between the experimental and control plots, two years after tent removal (ANOVA: $F_{2,6}$= 1.807; p = 0.243), recovery of perennial turf algae was also not affected by the sterilization process.

Examination of microscopic stages

When examined under UV light with fluorescence microscopy, labelled microscopic stages of *M. pyrifera* and *P. californica* fluoresced blue, making them easily identifiable. Unlabelled thalli, in contrast, fluoresced red due to the excitation of their chlorophyll (Figure 2A). This allowed for easy differentiation between labelled and unlabelled stages. These microscopic stages were observed to be gametophytes. As they grew, the stain became diluted throughout their thalli and thereby decreasing in fluorescence intensity. However, they remained relatively easy to identify even after they produced sporophytes, with the female gametophytes fluorescing blue and the emerging sporophytes fluorescing red (Figure 2B).

The microscopic stages of *D. ligulata* that were labelled and outplanted to the field on slides, were easily distinguished from the microscopic stages of

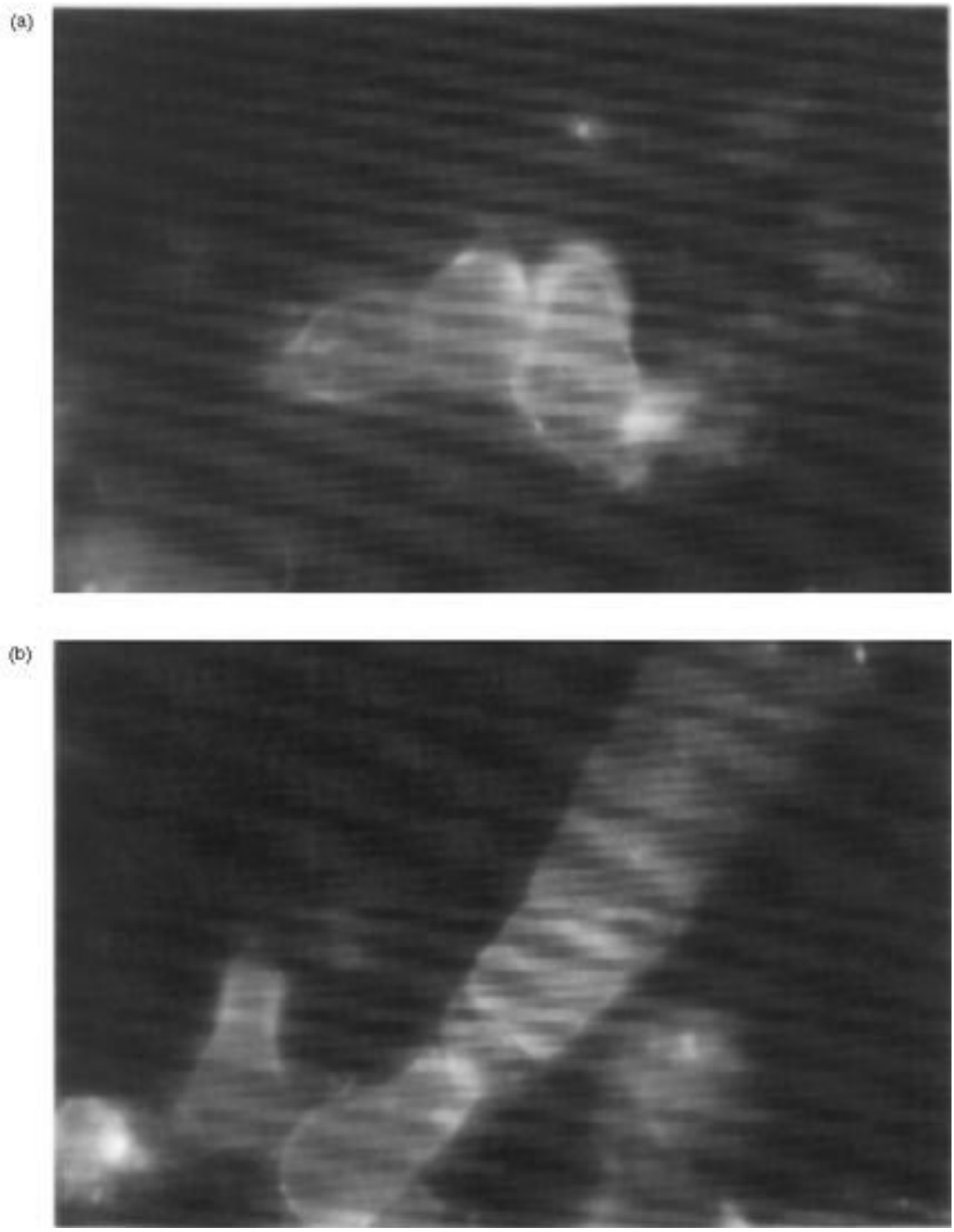

Figure 2. A. Fluorescently labelled *P. californica* gametophyte (blue) and unlabelled *P. californica* gametophyte (red) under UV light (red fluorescence is due to excitation of chlorophyll by UV light). Labelled gametophyte is 24 h older than unlabelled gametophyte. B. Single celled, labelled *P. californica* gametophyte (blue) after sporophyte (red) production (unlabelled multicellular tissue).

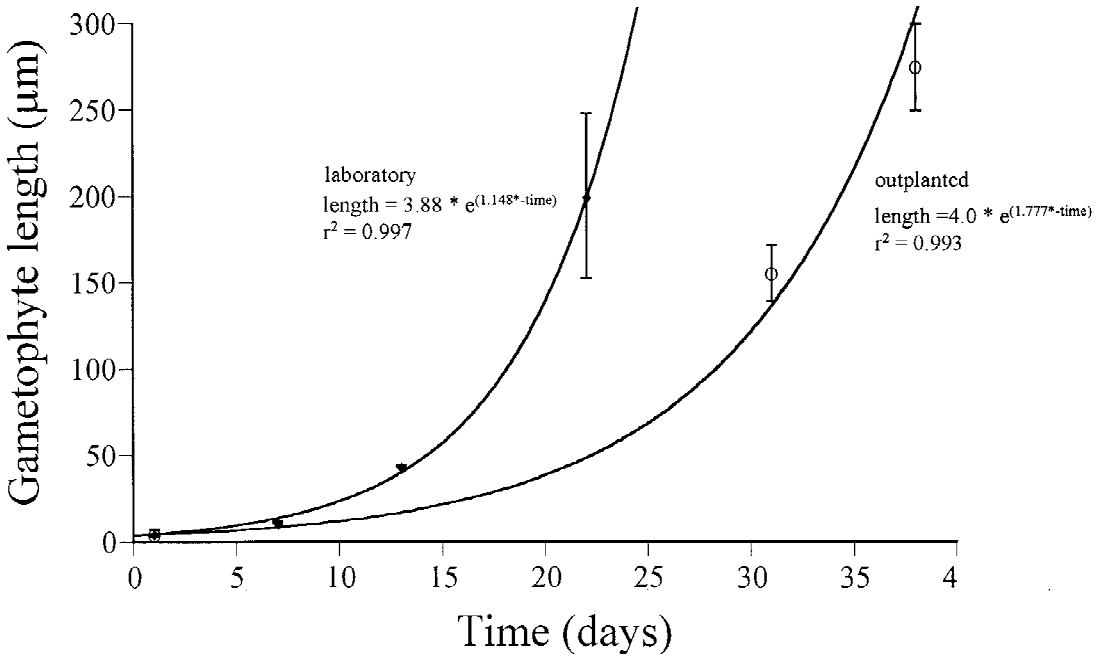

Figure 3. Growth of microscopic stages of *D. ligulata* (gametophytes) cultured in the laboratory and in the field.

other macroalgae that naturally settled on the slides when examined under UV light. The growth rates of the transplanted stages were estimated and compared with laboratory grown stages. Results indicated that *D. ligulata* microscopic stages exhibited exponential growth under both field and the laboratory conditions, with growth appearing slightly faster in the laboratory (Figure 3).

Discussion

With increasing appreciation of the contribution of banks of microscopic forms to the recruitment of natural populations of marine macroalgae, the ability to directly observe these microscopic stages in the field is becoming more important. This, however, has been problematic for subtidal species that occur on solid rock surfaces. Here, watertight tents provided an efficient way to remove the microscopic algal stages from these substrata using common household bleach. These tents allowed high concentrations of bleach to remain in continuous contact with the substratum for at least two days, thereby killing all the resident microscopic algae. By preventing the dilution of the sterilizing agent into the surrounding seawater, these tents allow for the use of a variety of water-soluble chemicals that have been available for use in intertidal habitats, e.g. formalin, phosphoric acid or alcohol. Also, since these chemicals are water-soluble, they will not leave toxic residues that may affect future settlement of algal propagules.

Fluorescent staining of algal propagules has been used to identify certain species and examine their propagule growth. The results from this study suggest that this technique can be used as a powerful tool in studying algal microscopic stages in the field. Here, it allowed for the identification of known thalli and for the precise measurement of their growth rates in the field. It can, therefore, be used to examine growth of algal microscopic stages under a variety of environmental conditions. This can be done *in situ*, whereas previously, growth of microscopic stages in various field conditions has typically been inferred from laboratory studies. This technique remained effective at identifying the microscopic stages even after production of new sporophytes, which greatly increases confidence in its ability to precisely identify labelled thalli throughout the various stages of their development. These different tools, when used together, should greatly increase our ability to examine the ecology and importance of microscopic stages to the recruitment of subtidal populations of marine macroalgae in the field.

Acknowledgements

I thank M. Foster for advice throughout this project. I also thank my field assistants: R. Clark, J. Downing, C. Roberts and especially S. Lamerdin who helped haul my tents to and from my study sites. I would like to extend my warmest appreciation to S. Tanner for her help with the fluorescence microscopy and E. Danner for his help with computer generated graphics. E. Danner, A. Boxshall, M. Foster and R. Walder reviewed this manuscript and offered helpful criticism. This project was funded in part by grants from The Dr Earl H. and Ethyl M. Meyers Oceanographic and Marine Biology Trust and the Packard Foundation.

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