

# Seeding nets with neutral spores of the red alga *Porphyra umbilicalis* (L.) Kützting for use in integrated multi-trophic aquaculture (IMTA)

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## Abstract

Nets in traditional *Porphyra* mariculture are seeded with conchospores derived from the conchocelis phase, and spend a nursery period in culture tanks or calm coastal waters until they reach several centimeters in length. Some species of *Porphyra* can regenerate the foliose phase directly through asexual reproduction, which suggests that the time, infrastructure, and costs associated with conchocelis culture might be avoided by seeding nets with asexual spores. Here, we present work from a short-term mariculture study using nets seeded with asexual spores (neutral spores) of a native Maine species of *Porphyra*. *Porphyra umbilicalis* (L.) Kützting was selected for this proof of concept research because of its reproductive biology, abundance across seasons in Maine, and evidence of its promise as a mariculture crop. We studied the maturation, release, and germination of the neutral spores to develop an appropriate seeding protocol for nets, followed by development of a nursery raceway to provide an easily manipulated environment for the seeded nets. Neutral spores were produced throughout the year on the central Maine coast; however, there was a temporal variability in the number and survival of released neutral spores, depending upon thallus position in the intertidal zone. Small thalli were strictly vegetative, but most thalli reproduced by neutral spores; sexual reproduction was absent. Neutral spores germinated quickly at 10 and 15 °C, but germination was delayed at 5 °C. Unlike some algal zygotes and spores, neutral spores of *P. umbilicalis* required light to germinate; however, irradiances of 25 and 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  were equally sufficient for germination. Rafts of seeded nets were deployed in Cobscook Bay, Maine, at two distances from salmon aquaculture pens and at a control site on a nearby, fallow aquaculture site (no salmon). There was no difference in nitrogen content of harvested thalli; however, both the density and the surface area of harvested thalli were different among the sites. The possible causes of these differences are discussed in the context of potential use of *P. umbilicalis* in IMTA.

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## 1. Introduction

Commercial mariculture of *Porphyra* has changed relatively little since the innovations (Tseng, 1981a) prompted by Drew's (1949) elucidation of this alga's life history. Strain selection has improved the yields and

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quality of harvested *Porphyra*, and cold-storage of seeded nets permits an extended growing season (Lobban and Harrison, 1994). However, nets are still seeded for commercial production with conchospores derived from the sporophytic phase, which is called conchocelis. The seeded nets go through a nursery period in culture tanks or calm coastal waters, often until the germlings reach several centimeters in length (Miura, 1975; Tseng, 1981a; Sahoo and Yarish, 2005). After the nursery period, the nets are moved to coastal waters, where they are harvested 6–8 times for up to 120 days. This requires extensive and expensive cultivation and maintenance of the conchocelis, even though the economically important gametophytic blade is grown for only 3–5 months of each production year. However, some species of *Porphyra* can regenerate the foliose phase directly through asexual reproduction (Drew, 1954; Hawkes, 1977; Kapraun and Luster, 1980; Hymes and Cole, 1983; Avila et al., 1986; Nelson et al., 1998; Wiencke and Clayton, 1998; Notoya and Nagaura, 1999; Brodie and Irvine, 2003). This suggests that the time, infrastructure, and cost associated with conchocelis culture could be avoided by seeding nets with asexual spores (Hafting, 1999; Notoya, 1999; Mizuta et al., 2003; He and Yarish, 2006). Indeed, the asexual spores of *P. yezoensis* Ueda (i.e. archeospores) are just beginning to be used effectively in commercial-scale nori culture in China (X. G. Fei, unpublished).

Eutrophic inputs of nitrogen and phosphorus from finfish farming can be reduced using an integrated approach that combined aquaculture of marine macroalgae (sea vegetables) with finfish (Folke et al., 1994; Krom et al., 1995; Fei et al., 1998; Chopin et al., 2001; Yang and Fei, 2003; Neori et al., 2004). The marine macroalgae benefit from the polyculture with finfish because the algae require the nitrogen and phosphorus that are waste products in finfish aquaculture from uneaten food and fish excretions ( $\text{NH}_4^+$ ). The aquaculture industry has already reduced the amount of uneaten food that contributes to waste by monitoring fish feeding with underwater video; feeding is stopped when fish are satiated (D. Morang of Cooke Aquaculture Inc., personal communication). However, the most efficient system will still add nutrients to the water column from some proportion of food waste and fish excretions. Native northwestern species of *Porphyra* are one target for integrated multi-trophic aquaculture (IMTA).

Here we evaluate the potential for use of the native Maine species *Porphyra umbilicalis* (L.) Kützinger in mariculture by seeding nets with its asexual spores (neutral spores sensu Nelson et al., 1999). At least seven

species of *Porphyra* are native to the Gulf of Maine (Sears, 2002; Klein et al., 2003), and more species are likely to be described from the northwestern Atlantic (Broom et al., 2002; Neefus et al., 2002; Klein et al., 2003). Additionally, *P. yezoensis* and *P. suborbiculata* Kjellman, two invasive species from Asia, were reported recently in the northwestern Atlantic, although their distribution is limited (Broom et al., 2002; Carlton, 2004; West et al., 2005). Except for *P. umbilicalis*, all of these species are seasonally restricted algae with confirmed sexual life histories in the northwestern Atlantic (Yarish et al., 1998; Sears, 2002; Neefus et al., 2002). *Porphyra leucosticta* Thuret (Holmes and Brodie, 2005), *P. purpurea* (Roth) C. Agardh (Bray et al., 2006), *P. suborbiculata* Kjellman (e.g. Freshwater and Kapraun, 1986; Nelson et al., 1998), and *P. yezoensis* (Notoya, 1999) reproduce via sexual and asexual pathways. In contrast, *P. umbilicalis* is present year-round and appears to reproduce only asexually in the northwestern Atlantic (see Results), although it reproduces sexually and asexually in Europe (Conway, 1964a,b; Kornmann and Sahling, 1991; Brodie and Irvine, 2003). We chose to use *P. umbilicalis* for this proof of concept research on seeding nets with asexual spores because of its reproductive biology, abundance across seasons in New England, and evidence of its promise as a mariculture crop (Kraemer et al., 2004; West et al., 2005; Blouin et al., 2006; Carmona et al., 2006). We collected thalli and neutral spores throughout the year along the Maine coast to determine whether there were seasonal effects on the presence of reproductive thalli and the viability of neutral spores. Next, optimal conditions for neutral spore germination were investigated in the laboratory to develop a seeding protocol. We also focused special attention on developing a nursery system for the seeded nets to provide an easily manipulated environment in which epiphytes and grazers could be controlled and where germlings could be monitored. Here we demonstrate that nets can be seeded with asexual spores, grown as germlings in a raceway system, and then deployed in an integrated mariculture setting in Cobscook Bay, Maine, U.S.A.

## 2. Materials and methods

### 2.1. Field collections

Monthly collections of *P. umbilicalis* were made at Blueberry Hill, Schoodic Point, Maine (44 ° 20' 20.39" N, 68 ° 2' 42.03" W) from the high- (0.0 to –0.05 m MHT); the high-mid- (–1 to –2 m MHT); and mid-

intertidal zones (–2.5 to –3.5 m MHT). Thalli were randomly collected along horizontal transects within each zone to locate 10 collection positions. At each collection position, a 45 cm long string with 10 knots was dropped, and any thallus under a knot was collected. The thalli were pressed on herbarium paper, and the reproductive state of each thallus was determined by viewing the marginal sections on a Nikon Optiphot 2 microscope.

## 2.2. Temporal and spatial effects on neutral spores in the field

*P. umbilicalis* thalli were collected as described above and trimmed in the field with scissors to obtain ~1 cm wide margins. The thalli were loosely wrapped in paper towels, placed on ice, and transported back to the lab within 2 h. Neutral spores were obtained by air-drying the thalli in an Intellus I-35LLVL incubator (Percival Scientific, Perry, IA) fitted with 20-watt cool white fluorescent bulbs (Osram Sylvania, Danvers, MA) at 20- $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 10 °C for 30 min (~3:1 damp: dry ratio). After drying, the thalli were rinsed in a plastic container with 750 mL autoclaved seawater (SW) for 1 min to remove spores that had already been released but still clung to the thalli margins. After shaking the container briskly for 30 s, the SW was discarded, and the *P. umbilicalis* was spun in a salad spinner lined with paper towels to remove excess moisture and spores. The margins were immediately placed into a glass beaker containing 250 mL SW enriched with Provasoli's solution (SW–ES), and shaken (1 h, 90 RPM) on a VWR orbital shaker (model #980001, VWR Scientific Chester, PA.), at 50  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 10 °C. At the end of 1 h, the margins were strained out of the spore suspension with 50  $\mu\text{m}$  Nitex cloth (E M Mfg. and Instrument Co., Nanuet, NY). All Petri dishes used in neutral spore experiments were pretreated with SW to remove static surface charges that could cause the neutral spores to burst when they settled. The spore suspension was quantified by placing a 10 mL aliquot into a sterile 60  $\times$  15 mm polystyrene Petri dish with a 2 mm grid (Corning Glass, Corning, NY). All spore counts were done on a Zeiss IM-35 inverted microscope (100 $\times$ ). All counts were conducted on randomly selected 16 mm<sup>2</sup> blocks of the Petri dishes. Petri dishes ( $n=5$ ) containing 10 mL of spore suspension were placed haphazardly into incubators in still conditions at 10 °C, 50  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 12:12 (L:D). The number of spores released  $\text{g}^{-1}$  dry wt (DW) and survivorship of spores were determined by the methods described above after 1 wk.

## 2.3. Germination experiments

The collection and preparation of *P. umbilicalis* used in these germination experiments were as follows: 5–20 g of reproductively mature *P. umbilicalis* was haphazardly collected from the mid-intertidal zone. The thalli were collected just after emersion during early morning low tides, loosely wrapped in paper towels, placed on ice, and transported back to the lab within 2 h. Neutral spores were obtained by the methods described above. The percentage of germinated spores was determined over a 7 d period for each experiment.

### 2.3.1. Effect of water motion on neutral spore germination

The Petri dishes for all treatments were placed in still conditions for 4 h (100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  10 °C) to allow spores to settle ( $n=6$ ). Then the Petri dishes were rinsed with fresh SW–ES to remove unattached spores. The Petri dishes were randomly assigned to shaken or calm conditions in 2 different incubators, 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  10 °C, 12:12 (L:D). Shaken treatments were placed on VWR orbital shakers (model # 980001) at 60 rpm.

### 2.3.2. Effect of irradiance on neutral spore germination

The following irradiance levels were tested to examine the effect of irradiance on the rate of spore germination: 0, 25, and 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Petri dishes ( $n=6$ ) containing 10 mL of spore solution were placed in still conditions, 10 °C, 12:12 (L:D). The dark treatments were wrapped in aluminum foil and destructively sampled (18 dishes total).

### 2.3.3. Effect of temperature on neutral spore germination

The following temperatures were tested to examine their effect on neutral spore germination: 5, 10, 15 °C. Petri dishes containing 10 mL of spore solution were placed in still conditions at 75  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 12:12 (L:D). The Petri dishes ( $n=3$ ) were assigned randomly to individual incubators.

## 2.4. Raceway construction

A 5 m<sup>3</sup> raceway (Fig. 1) was constructed as a nursery environment for seeded nori nets at the University of Maine's Center for Cooperative Aquaculture Research (CCAR) in Franklin, ME. The raceway was designed to accommodate enough nets to establish a small commercial-scale operation (~667 m<sup>2</sup> or 1[Chinese] mu). It consisted of a deck that was constructed of 5 cm by 20.3 cm lumber, which was then sheathed in 2 cm thick



Fig. 1. Image showing the layout and construction of the raceway (top) and method of suspending the nets in the raceway using PVC pipes (bottom). 1) Deck; 2) sheathing; 3) curved radius; 4) center divider; 5) make-up inflow; 6) standpipe; 7) shading.

plywood. The 30 cm high sidewall and center divider of the raceway were also covered in 2 cm plywood except at the curved ends. The 1.2 m (radius) curved ends of the raceway were built out of 3 layers of 0.6 cm plywood. This thin and flexible plywood formed the 1.2 m radius curve without breaking. The drain was countersunk 2 cm to aid periodic draining of the raceway, and the drain hole was fitted with a 10 cm diameter, schedule 40 bulkhead fitting. The drain was fit with a bottom-draining standpipe to maintain a constant water level. Construction materials are all available from local home improvement stores and plumbing suppliers in the U.S. (e.g. The Home Depot). Two layers of 283 g fiber-cloth

and polyester resin (Kardol LLC, Lebanon, Ohio) were used as a base layer in the raceway for waterproofing and structural integrity. Waterproofing was finished with 2 additional layers of white Gel-Coat (Kardol LLC, Lebanon, Ohio), which also provided ~25% reflectance of incident light. The raceway was then covered with 2 layers of 60% greenhouse shade-cloth (Farmtek Inc., Dyersville, IA) suspended 2 m above the raceway on wood framing to reduce irradiance and solar gain. Water temperature and other environmental conditions were maintained with 50 L min<sup>-1</sup> make-up flow with seawater pumped from Taunton Bay, ME (sand filter pretreatment) to further counter the effects of solar gain.

We maintained a circular flow in the raceway with three medium-head aquarium pumps (Mag Drive MD5, 700 gal h<sup>-1</sup>, Danner Mfg. Inc, Islandia, NY).

### 2.5. Net-seeding and nursery stage

Nori nets (20 cm<sup>2</sup> mesh size, 1.7 m×1.7 m) were obtained from Dr. P. He, at Shanghai Fisheries University, Shanghai, China. The nets were made from one line with three twisted components: a polyethylene thread for spore attachment, and two polypropylene threads to increase strength. In preparation for seeding, the nets were washed three times in commercial washing machines in warm water without detergent and air-dried. This was done to remove any harmful residues left over from the manufacturing process.

*P. umbilicalis* for net-seeding was collected from the open shore at Dark Harbor, Grand Manan Island, New Brunswick, Canada (44.75°N 66.85°W), on July 11, 2005 (voucher deposited at the University of Maine Herbarium #DH071105 1–6) and kept at 4 °C in the dark for 36 h before use. We used material from Grand Manan because viable spores could not be obtained at our Schoodic Point field sites in summer (see below). Wild material with a well-defined deep red margin was selected for seeding, because such a margin was indicative of maturing neutral sporangia. We screened 1.0 kg of damp whole thalli to remove grazers by rubbing the *P. umbilicalis* against a plastic screen with a 1 cm<sup>2</sup> sized mesh and then air-dried the material in the shade for 1 h, which killed remaining amphipods. These “semi-dried” (~3 times dry weight) *P. umbilicalis* blades were placed in a plastic tub with 20 L of 20 µm-filtered seawater (SW) for 1 h at 16 °C. After 1 h, the *P. umbilicalis* thalli were removed from the spore suspension using plastic screen. The number of spores was quantified using a special counting chamber for spore enumeration (IOCAS, (1978)). This chamber consisted of a standard microscope slide with a piece of 2 mm thick white Plexiglas glued to its surface. A 0.4 mL well was created by drilling a 16 mm diameter hole in the plastic. The number of spores mL<sup>-1</sup> was determined by the average of 3 chambers, counting five randomly selected fields (100×) per chamber and using a multiplier for the chamber to determine the number of spores mL<sup>-1</sup>. Following spore enumeration, thirty 1.7×1.7 m nets were seeded with 1.8×10<sup>9</sup> neutral spores (see below). Spore density and attachment were determined by observing separate pieces of nori net that was included in the seeding process.

The cleaned and dried nets were individually folded like an accordion and placed side-by-side in a

1×2×0.3 m plastic tub in a 15 °C walk-in, air-cooled room. The spore suspension was poured over the nets, and they were agitated by hand at 5 min intervals for 1 min. After 30 min, 20 µm-filtered SW was added to a depth that just covered the nets. The nets were left undisturbed for 3 days in the following conditions: 15 °C; 60 µmol photons m<sup>-2</sup> s<sup>-1</sup>; 12 h:12 h [L:D] (4, 8 ft 110 W, high-output, cool white fluorescent bulbs, GE Lighting, Fairfield, CT). After 3 days, the nets were moved into the raceway under natural sunlight (15–19 °C, 140 µmol photons m<sup>-2</sup> s<sup>-1</sup>=mid-day maximum). The nets were stretched between two PVC poles and suspended in the raceway (see Fig. 1). During the nursery phase, the nets were fertilized daily in 2 h pulses with additions of fertilizers obtained from a local farm supplier: NH<sub>4</sub>NO<sub>3</sub> (to produce a final concentration of 2 mM N in the raceway) and soluble phosphorous (to a final concentration of 0.2 mM P in the raceway). The make-up flow was turned off when nutrients were added, so that the raceway temporarily functioned as a closed system. When the seawater temperature increased by 1 °C, or after 2 h, the make-up seawater flow was restarted.

Nets were hung in the shade every six days (weather permitting) to control the growth of chain-forming diatoms and cyanobacteria by desiccation. A low density of diatoms was kept on the nets to inhibit the settlement of green algal spores and to control the growth of uniseriate green algae. The nets were kept for 7 weeks in the raceway before being transported to Cobscook Bay while wrapped in cloth on ice to avoid heat shock.

### 2.6. Grow-out phase in Cobscook Bay

The framework for the raft consisted of two 40 m long lines (1.6 cm diameter polypropylene pot warp) that were kept ~2 m apart with 2.5 cm diameter PVC pipe ribs that were placed two net widths apart along their length (~3.5 m). Nine nets were quickly tied into each raft while it was stretched out on the shore by tying each corner of the net to the frame with several half hitch knots so that each net was taut. The nets seeded with *P. umbilicalis* were deployed at two sites where salmon farming occurs in Broad Cove (BC, 44° 54.4' N 67° 01.0' W) and at a site where there is no dissolved nutrient loading from salmon pens in Deep Cove (DC, 44° 53.8' N 67° 0.0' W) on lease sites held by Cooke's Aquaculture Inc. (Black's Harbor, New Brunswick, CA). Rafts of nets in BC were placed 30 m northeast (BCNE) of the salmon pens and also 200 m southeast (BCSE) of the pens (Fig. 2). Deep Cove was a control

for the salmon-associated nets. We collected two sets of surface water samples (data not shown) in fall 2004 during low tide at BC that showed that the BCNE site had higher nutrient levels than the BCS site, but it is important to note that the higher nutrient site did not have nutrient levels that represent any significant level of eutrophication. Our intent in selecting these sites was to observe whether growth rates were affected by distance from the salmon pens, but site constraints and other logistical problems (e.g. circulation patterns, money) prevented the use of a properly replicated experimental design, which would have required rafts at more than a single salmon pen. Results are not tested with ANOVA for this reason (i.e. pseudo-replication).

At each site, a single 2×20 m floating raft was oriented parallel to the dominant flow based on information from Cooke Aquaculture (north–south for both BC rafts and east–west for DC). The rafts were moored with one anchor at either end. Two 60 cm mooring buoys were placed at each end of the raft where the connection between the raft and the mooring lines was made. Small floats with 1 m long line were placed at each polyvinyl chloride rib to keep the negatively

buoyant raft at a constant depth of 1 m. When the nets needed to be dried to reduce diatoms, the raft was untied at one end, folded and hauled into a skiff, and hung from a nearby pier to dry in the shade for 2 h. The nets were dried 3 times over 4 weeks.

## 2.7. Harvesting and assessment

Factors that were analyzed to determine differences between sites included: blade morphometrics, C and N analyses of thalli, and environmental and nutrient analyses of seawater samples from Cobscook Bay.

### 2.7.1. Blade morphometrics

Eighteen randomly selected, 10 cm long pieces of net line were collected from each raft. All thalli were removed with forceps under a dissecting microscope and the surface area of each thallus was determined with Image J® freeware ([www.nih.gov](http://www.nih.gov)). The total surface area was calculated for blade morphometrics, rather than mean frond length as reported in previous investigations (Holmes and Brodie, (2004; West et al., 2005) because of morphological plasticity.

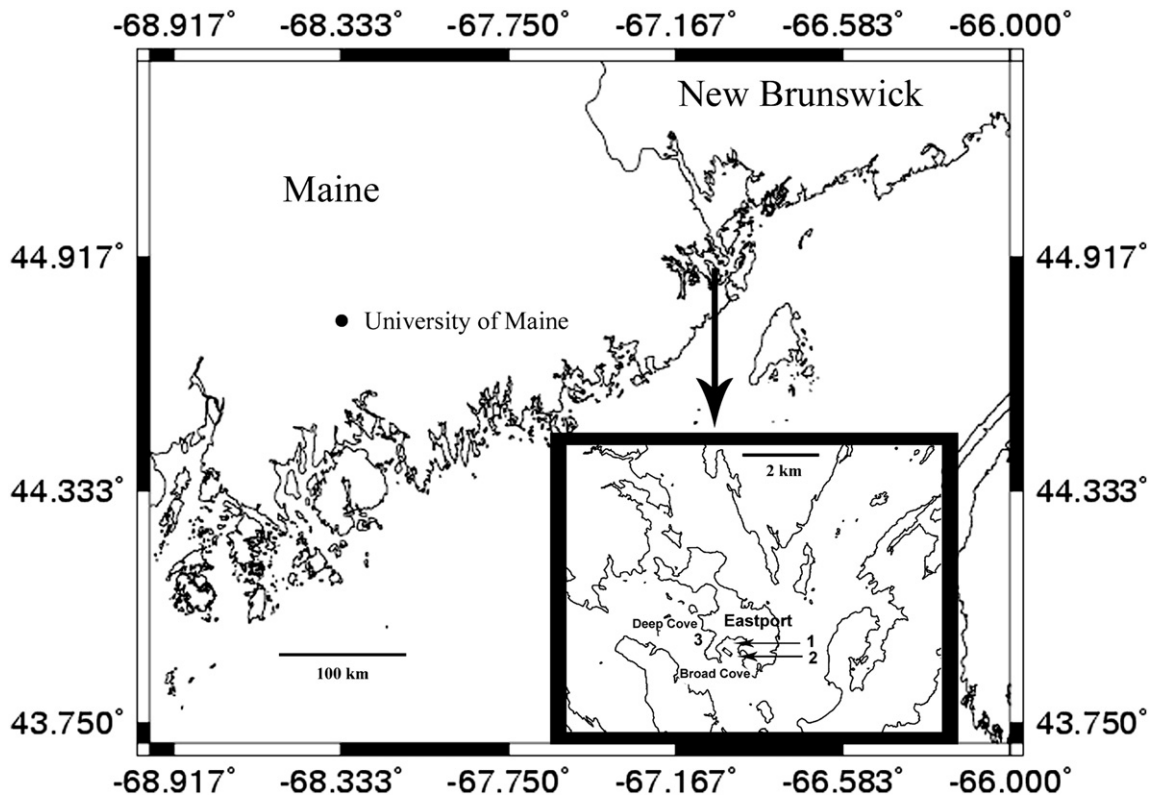


Fig. 2. Map of the downeast Maine coast. Inset of Moose Island, Eastport, showing the location of the experimental rafts (1, BCNE; 2, BCSE; 3, DC) in Broad and Deep Coves.

2.7.2. C and N analysis

All of the largest blades were removed from randomly selected pieces of net on each of the three rafts (using a random numbers' table and a grid). Each group was blotted on paper towels and transported to the laboratory on ice. All material was dried at 65 °C in a Fisher Isotemp drying oven (Fisher Scientific, Hampton, NH) for 48 h. The carbon and nitrogen of the blades were analyzed on a Perkin Elmer 2400 CHN Analyzer (Perkin Elmer, Inc., Wellesley, MA) at the University of Maine's Darling Center (Walpole, ME).

2.7.3. Environmental and nutrient data (seawater temperature,  $NO_2^- + NO_3^-$ ,  $NH_4^+$ ,  $PO_4^{3-}$ )

At the beginning, middle, and end of the experiment, water samples were collected from ~1 m depth (net-level), particularly to determine whether there was a

stronger  $NH_4^+$  signal near the salmon pens from fish excretions. Triplicate samples were collected every 2 h over a complete tidal cycle (12 h) from each site. All collections were made with buckets rather than Niskin bottles. The buckets were used because they allowed one investigator to obtain all of the water samples at the three rafts during the tidal sampling period. The SW was filtered with 0.22  $\mu m$  acetate filters (Osmonics Inc., Minnetonka, MN) into 30 mL scintillation vials. The vials were frozen within 1 h of collection, which stops the conversion of  $NH_4^+$  to other forms of nitrogen. Nutrient analysis was performed using a Bran and Lubbe AA3 Auto Analyzer (SPX Process Equipment, Inc., Delavan, WI). Water temperature was taken directly from Station PSBM1 – 8410140 – Eastport, ME (NOAA, National Buoy Data Center), which is located near the mouth of Cobscook Bay.

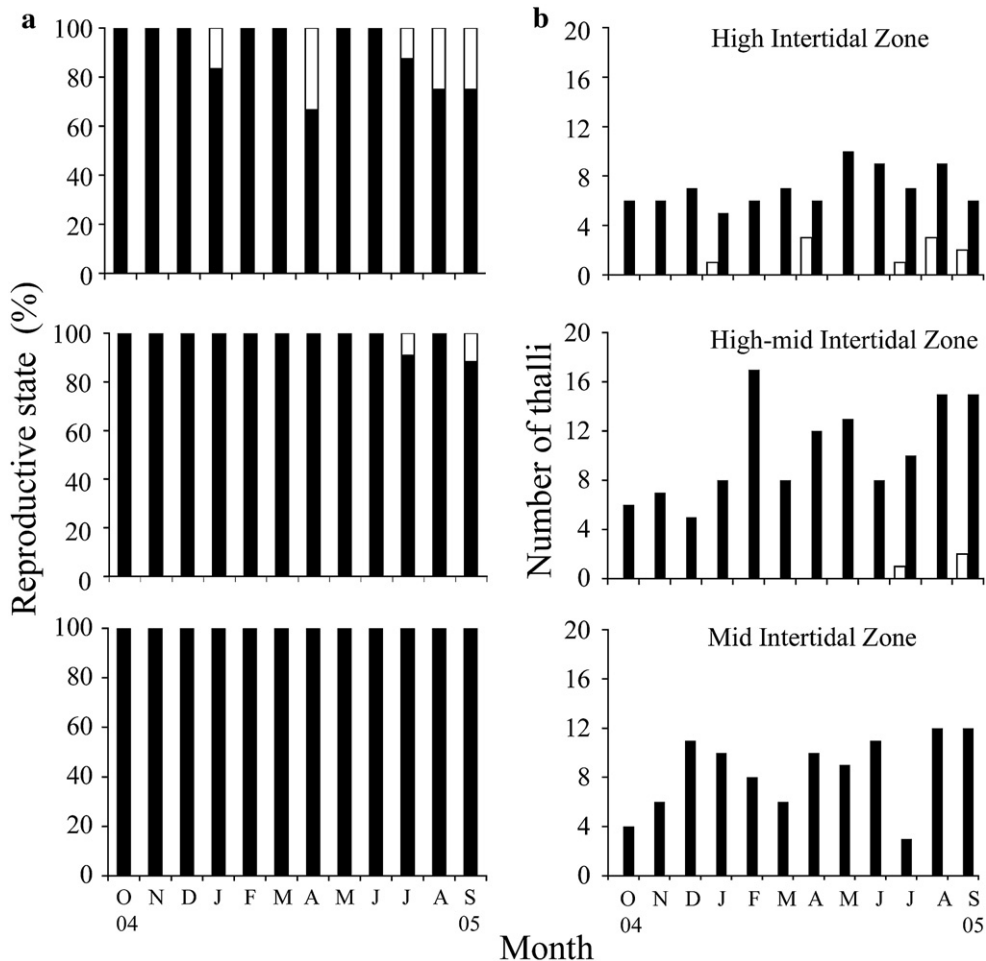


Fig. 3. Monthly reproductive pattern (neutral sporangia) across tidal heights for *Porphyra umbilicalis*, shown as the percentage (a) and the number (b) of vegetative (white) and asexually reproductive thalli collected on transects at Schoodic Point, Maine (see Materials and methods). No sexual reproduction was observed.

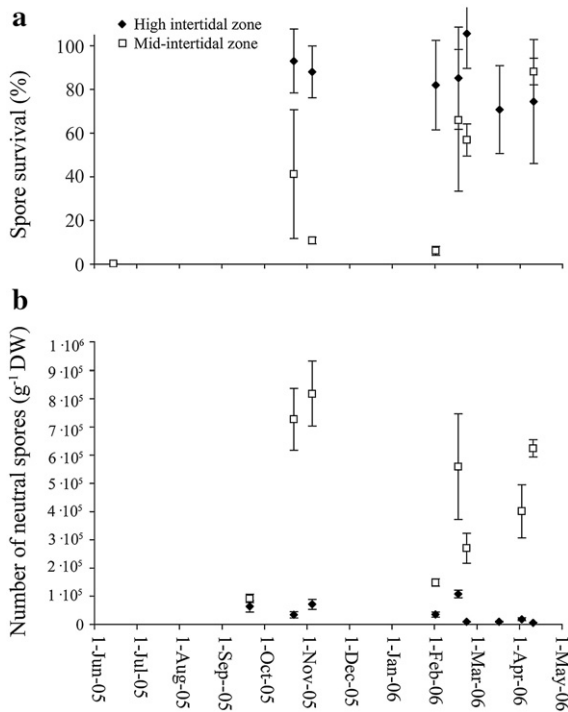


Fig. 4. Seasonal observations of (a) neutral spore survivorship and (b) numbers of neutral spores released (mean  $\pm$  SD).

### 2.8. Statistical analysis

The statistical analyses were carried out using SYSTAT 11 software package (Systat Software, Inc., San Jose, CA). A one-way analysis of variance (ANOVA) was used to investigate temporal and spatial effects on neutral spores in the field. An analysis of covariance (ANCOVA) was used to determine the differences in the percentage of neutral spores germinated in the germination experiments.

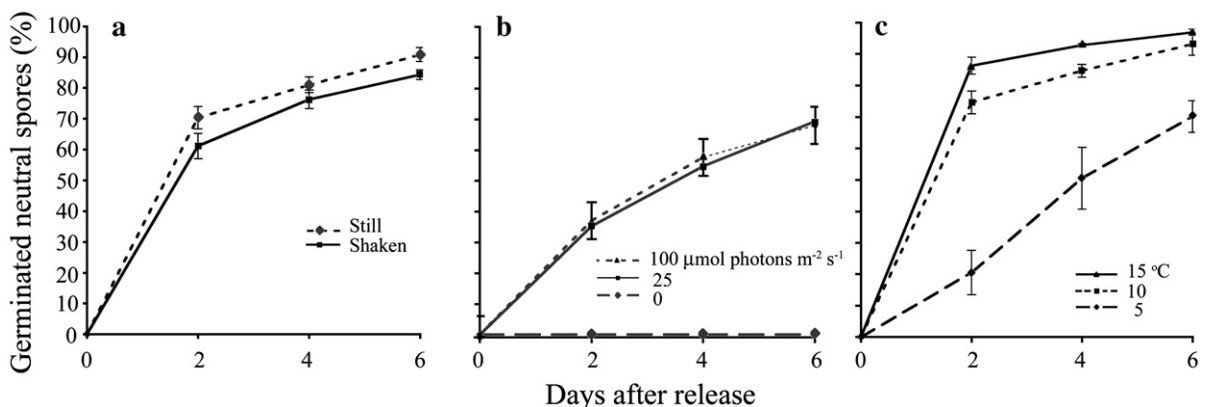


Fig. 5. The effect of water motion (a), irradiance (b), and temperature (c) on neutral spore germination in *Porphyra umbilicalis* (mean  $\pm$  SD).

## 3. Results

### 3.1. Field collections

Reproductive thalli were found throughout the year (Fig. 3) and ranged in length from 1 to 44 cm. The majority of purely vegetative thalli were collected in the high-intertidal zone, and were all  $\leq 4$  cm long.

### 3.2. Temporal and spatial effects on neutral spores in the field

The number of neutral spores released from thalli was dependent upon the position of the thallus on the shore (i.e. tidal height). Spore release from thalli collected in the mid-intertidal zone was 2 orders of magnitude higher than from thalli in the high-intertidal zone ( $F_{(1,86)} = 106.21$ ;  $p = 0.000$ ). There was no effect of tidal cycle (i.e. spring versus neap) on amount of release at either tidal height. There was a large amount of variability in the release of spores from both intertidal zones; however, the thalli growing in the high-intertidal zone showed less variability (Fig. 4). Survivorship of neutral spores after release was significantly higher in neutral spores from high-intertidal thalli ( $F_{(1,68)} = 25.732$ ;  $p = 0.000$ ).

### 3.3. Germination experiments

#### 3.3.1. Effect of water motion on neutral spore germination

Germination of neutral spores was slightly higher in calm conditions than under shaken conditions ( $F_{(1,15)} = 17.91$ ;  $p = 0.001$ ; Fig. 5). Day=0 was removed from the analysis because we did not collect data on the onset of germination in either treatment.



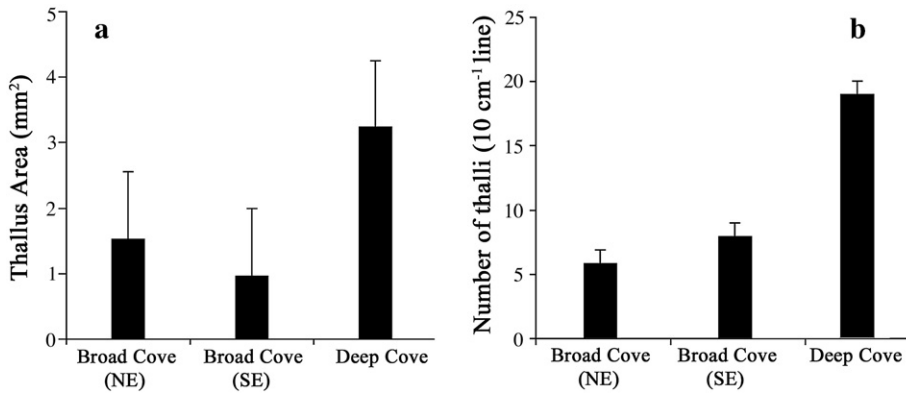


Fig. 6. Surface area of *Porphyra umbilicalis* thalli (a) and the number of thalli 10 cm<sup>-1</sup> of net line at each of the three sites (b, mean±SE).

3.3.2. Effect of irradiance on neutral spore germination

Spores failed to germinate in darkness and almost all of the spores in the dark treatment died by the end of the experiment; therefore, irradiance levels=0 were omitted from the analysis. ANCOVA analysis showed that there were no differences in germination between the 25 and 100 μmol photons m<sup>-2</sup> s<sup>-1</sup> treatments over the week (Fig. 5). Day=0 was removed from the analysis.

3.3.3. Effect of temperature on neutral spore germination

ANCOVA analysis showed that the percentage of germinated neutral spores was highest in the 15 °C treatment over the period investigated, and the 5 °C treatment caused lower and slower germination than either the 10 or 15 °C treatments ( $F_{(2,21)}=3.86, p=0.037, F_{(2,21)}=16.000, p=0.000$  respectively; see Fig. 5). Day=0 was removed from the analysis.

3.4. Net-seeding and nursery stage

Neutral spores attached and germinated within 5 days of seeding, but germlings grew slowly thereafter. Neutral

spore density was determined to be 1.3 (±1.2, SD) neutral spores mm. The nets were periodically fouled by diatoms, cyanobacteria, and filamentous green algae (*Ulothrix* sp.) during the nursery phase, but periodic desiccation was sufficient to control the growth of fouling organisms. As the summer progressed, the temperature in the raceway warmed to 19 °C.

3.5. Harvesting and assessment

At the time of deployment of nets into Cobscook Bay (6 September 2005), the largest germlings were 1 mm in length. Nets had to be removed from Cobscook Bay on 30 September 2005 because of the onset of the dragging season for sea urchins.

3.5.1. Blade morphometrics

The average thallus size was larger at Deep Cove than at either of the Broad Cove sites (Fig. 6). Average area of thalli for all sites (mm<sup>2</sup>±SE) was: 0.97±1.01, BCSE; 1.54±1.03, BCNE; and 3.24±1.01, DC. Average number of thalli for all sites (per 10 cm<sup>-1</sup> of net

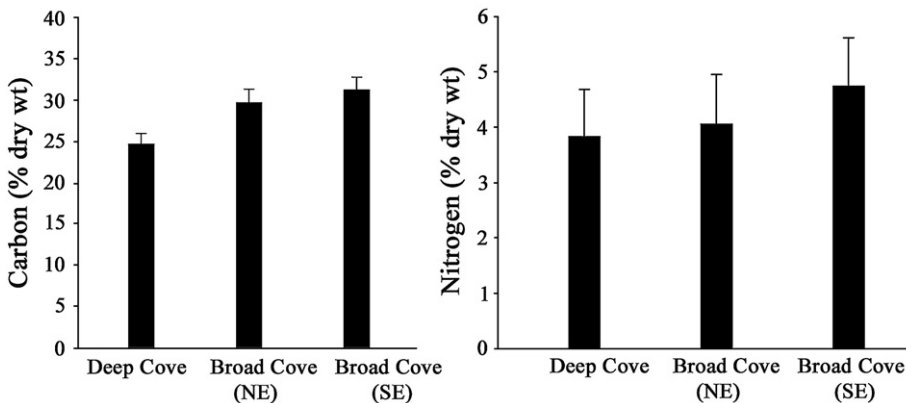


Fig. 7. C:N analysis of *Porphyra umbilicalis* from each of the sites in Cobscook Bay (mean±SE).

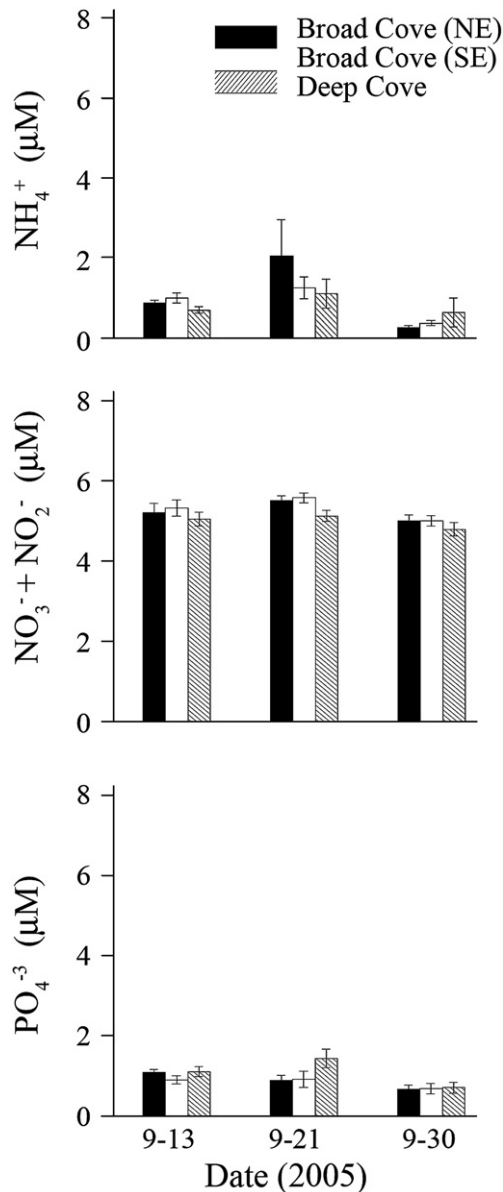


Fig. 8. Seawater nutrient data for each site grouped by date of collection (mean±SE).

line ±SE) was:  $8.0 \pm 1.02$ , BCSE;  $5.9 \pm 1.02$ , BCNE; and  $19.0 \pm 1.01$ , DC. The density of thalli on nets was higher at the Deep Cove site (Fig. 6).

### 3.5.2. C and N analysis

The nitrogen content of thalli was similar among the different rafts; however, there was less carbon in the Deep Cove material (Fig. 7). The % N±SE for each of the rafts was:  $4.73 \pm 0.86$ , BCSE;  $4.05 \pm 0.88$ , BCNE; and  $3.82 \pm 0.84$ , DC. The % C±SE for each of the rafts

was:  $31.35 \pm 1.49$ , BCSE;  $29.80 \pm 1.59$ , BCNE; and  $24.76 \pm 1.25$ , DC.

### 3.5.3. Environmental/nutrient data

The water temperature in Cobscook Bay was constant at  $11.5 \text{ }^\circ\text{C}$  ( $\pm 0.5 \text{ }^\circ\text{C}$ ) during the experiment. Nutrients did vary over time, but not by location (Fig. 8). The lack of differences in overall nutrient concentrations between sites is also reflected in the percent nitrogen data for each of the three locations. Nutrient concentrations for September 2005 averaged over all sites ( $\mu\text{M}$  ( $\pm\text{SD}$ )) were:  $1.56$  ( $\pm 5.33$ )  $\text{NH}_4^+$ ,  $5.18$  ( $\pm 0.61$ )  $\text{NO}_2^- + \text{NO}_3^-$ , and  $0.93$  ( $\pm 0.50$ )  $\text{PO}_4^{-3}$ .

## 4. Discussion

Discussion of our results first requires clarification of the systematics of *P. umbilicalis* and closely related species. Three published studies used a putatively sexually reproducing strain of *P. umbilicalis* from the northwestern Atlantic Ocean (Chopin et al, 1999; Kraemer and Yarish, 1999; Carmona et al., 2006). At the time of those investigations, traditional morphological, seasonal, and reproductive diagnostic techniques were used to confirm species identity, but Neefus and co-workers (Neefus 2002; Klein et al., 2003) revealed that cryptic diversity exists in northwestern Atlantic species of *Porphyra* through molecular systematic studies. A RFLP assay was developed to distinguish between the cryptic taxa Teasdale et al., 2002), which include *P. birdiae* Neefus and Mathieson, *P. purpurea*, and *P. umbilicalis*. The ME40-6 strain used in Chopin et al. (1999) and Kraemer and Yarish (1999) was tested by the RFLP assay and confirmed to be *P. purpurea* (Blouin, 2006). The strain of '*P. umbilicalis*' used by Carmona et al. (2006) was not studied with molecular techniques and, unfortunately, it no longer exists as a collection, so we have used quotation marks when referring to this investigation below (per usage of Brodie et al., 1998). It is very unlikely that Carmona et al. (2006) used *P. umbilicalis* in their investigation. We believe the material was likely to have been *P. birdiae* or *P. purpurea*.

Our field collections showed that *P. umbilicalis* is asexually reproductive year-round, but the number and viability of neutral spores vary throughout the year. Release of neutral spores does not appear to be under the influence of the tidal cycle as in some other macroalgae (e.g. Ulva, Gordon and Brawley, 2004); however, in our observations there is temporal variability in the viability of neutral spores in *P. umbilicalis*. Temporal variability in spore quality was suspected when all of the spores

lysed within a day from a large release on June 15th 2005. The number of spores released for that date is an estimate, because we intended to use these neutral spores in development of net-seeding techniques. Subsequently, spores lysed from two other collections between June 15th and July 10th (S. H. Brawley and X. G. Fei, personal observation, data not shown). During the summer, *P. umbilicalis* is subjected to large fluctuations in temperature between days and over tidal cycles (up to 35 °C). Temperature stress coupled with increased irradiance levels and lower nutrients experienced in summer may have caused the decrease in viability recorded in June 2005. The higher survivorship in culture of neutral spores from high-intertidal thalli suggests that there are stress responses during spore maturation that are advantageous to neutral spore survival.

The range of irradiance levels tested here was intended to cover the range of illumination that can be economically produced in a large-scale seeding facility rather than those found in nature. The maximum irradiance levels from high-output white fluorescent fixtures currently used in land-based aquaculture are  $\sim 50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  at a distance of 0.5 m (2.6 m long, double-lamped units, 220 V). With this in mind, providing irradiance levels below  $50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  could reduce the cost of infrastructure for germinating nets seeded with neutral spores because our results show that neutral spores of *P. umbilicalis* germinate well in low light. Shading of spores by the nets themselves should not be a concern during the seeding or the nursery stage. Many algal zygotes and spores germinate in darkness and it is interesting that light is required for neutral spore germination in *P. umbilicalis*.

The nets seeded in this investigation required a fraction of the effort of traditional methods (Tseng, 1981a; Sahoo and Yarish, 2005) and, to our knowledge, this is the first time nets have been seeded with neutral spores from *P. umbilicalis*. One kilogram (fresh wt) of *P. umbilicalis* thalli released 1.8 billion spores in 1 h in our study. *P. umbilicalis* is reproductive year-round in Maine; however, the neutral spores obtained by rewetting of dried material were inviable under natural stress in summer. Care should be taken to obtain thalli for seeding that are a deep reddish-purple color.

The relatively short period of deployment in Cobscook Bay was necessary to avoid conflict with urchin draggers whose season began 1 October 2005. The short grow-out period in Cobscook Bay contributed to the small size of blades at harvest, but was long enough before the onset of urchin dragging season (1 October 2005) to assess the

success of the trial and determine problems in the next stage of pre-commercial research. Blades were healthy (i.e. normal shape and deep red color) at all three out-plant sites. The nitrogen content of the blades was within the reported range for nitrogen content for *Porphyra* (Chopin and Yarish, 1998; Kraemer et al., 2004). Recently, Carmona et al. (2006) reported 7% N dry wt for a cultured strain of '*P. umbilicalis*'. In contrast, *P. yezoensis* only reached a maximum of 5% N dry wt in the same culture conditions ( $300 \mu\text{M NH}_4^+$ ). The *P. umbilicalis* in this investigation reached over 4.7% N dry wt (BCSE). This is slightly higher than the maximum % N reported for this species in the only published field study (Hernández et al., 1993), which suggests that *P. umbilicalis* efficiently sequesters nitrogen at moderate-to-low nutrient levels as well as in high nutrient environments (Kraemer et al., 2004). The *P. yezoensis* gametophyte normally grows during winter when nutrients are typically higher than in summer (Tseng, 1984). It is possible that *P. umbilicalis* may have a growth advantage in lower nutrient environments over *P. yezoensis*, but this needs further investigation. We hypothesize that the lower % C at Deep Cove (DC) is an indication of faster growth at DC compared to the Broad Cove (BC) sites, leading to less storage of carbon and/or less deposition of cell wall material (Fogg, 1964; Dawes et al. 1974; Durako and Dawes, 1980). The seawater nutrients collected here do not suggest eutrophication; however, the levels were within the range required to sustain healthy growth of the algae (Tseng 1981b; Lobban and Harrison, 1994) at a time when nutrients are normally lower in the Gulf of Maine (Bisagni, 2003; Petrie and Yeats, 2000).

Maximum reported in vitro growth rates for *P. umbilicalis* are  $7\% \text{ d}^{-1}$  (wild-collected from the northeast Atlantic, Lüning, 1992) and  $13.1\% \text{ d}^{-1}$  (northwest Atlantic cultured strain, ME6-9), and *P. yezoensis* was found to be  $10.1\% \text{ d}^{-1}$  (Carmona et al., 2006). All of these measurements were obtained from experiments with adult tissue where the seawater nitrogen concentration was at least an order of magnitude higher than what is found in nature. Growth rates in this proof of concept trial averaged  $\sim 2.4\% \text{ d}^{-1}$  over the entire period of the investigation from seeding to harvest at the end of September. We hypothesize that the slow growth of *P. umbilicalis* was due to a higher than anticipated (and desired) water temperature in the raceway. Kraemer et al. (2004) showed that N uptake rate for a Maine strain of *P. umbilicalis* was highest at 10 °C. On Grand Manan Island, where the material for seeding the nets was collected (after we encountered poor spore quality in thalli from mid-coastal Maine),

water temperatures do not exceed 13 °C (PSBM1 — 8410140), whereas the raceway temperature was 16–18 °C for most of the nursery period with several days reaching 19 °C (data not shown). Hernández et al. (1993) reported that *P. umbilicalis* is a winter annual near the southern limit of its range in Europe. Its seasonality may be restricted by solar irradiance and temperature, and it is likely that our raceway conditions caused sublethal stress that slowed growth. Although *P. umbilicalis* in Maine is an aseasonal alga, it appears to be most abundant and healthy from late fall through spring when temperatures and irradiance levels are lower than in summer. Our field observations show that *P. umbilicalis* thalli become rubbery and green during summer in the intertidal zone on the Maine coast, and thalli lose flavor at this time (personal observation). Manipulating net depth in mariculture could correct this problem by exposing the *P. umbilicalis* to lower irradiance and temperatures than are found in the intertidal zone in summer, making it possible to use *P. umbilicalis* in mariculture year-round.

The differences in mean thallus size and density between sites in this study are unlikely to have resulted from variation in nutrient supply, because this was not significantly different across sites, and not higher in DC. It is unlikely that the differences in thallus size and density were due to physical disturbance because the highest amount of entangled drift occurred at DC. The differences in thallus size and density between DC and the BC sites may be affected by raft proximity to the salmon pens. Although placement of nets closer to finfish pens in previous studies showed increased growth (Chopin et al., 2001), that may be due to a greater availability of  $\text{NH}_4^+$  (Kraemer et al., 2004; Carmona et al., 2006), finfish pens provide a source population for biofouling of the nori nets (see below).

We hypothesize that the close proximity of the *P. umbilicalis* rafts in BC to the salmon pens led to increased diatoms, amphipods and detritus that were found on the nets, particularly at the NE treatment. The salmon pens are colonized by large standing populations of amphipods (including *Caprella* spp. and *Gammarus* spp.) and opportunistic species of algae. These organisms recruited to the BCNE nets within a few days of out-plant. In contrast, the raft in the DC treatment was nearly free of fouling organisms. Deployment of nets with germling *Porphyra* should be delayed until the thalli are of sufficient size (>1 cm length) to withstand the increased levels of herbivory and epiphytes near finfish pens (Brawley and Fei, 1987), and other measures need to be implemented to take advantage of integrated mariculture sites. For example, more frequent

desiccation of the nets will help to control these problems, and raft designs more appropriate for a location such as BC need to be developed. For example, a raft design from South Korea incorporates a boat-mounted mechanical arm that is capable of drying 100 m lengths of nets at a time with little effort (Sahoo and Yarish, 2005).

Nets seeded with asexually derived spores have a number of advantages. The use of neutral spores, as opposed to conchospores, eliminates the need to complete the sexual life history of *Porphyra*. This means that considerable time, expense and infrastructure necessary to maintain conchocelis cultures can be avoided. Labor costs are much greater in the United States than they are in many parts of Asia, and net-seeding with neutral spores may be cost-effective. Further, asexually generated blades from archeospores in *P. yezoensis* grow more quickly than those generated from conchospores (Li, 1984). It is possible that similar growth benefits for germlings may be present in the neutral spores of *P. umbilicalis*.

Raceways are widely used in microalgal culture (e.g. *Dunaliella*, *Spirulina*); however, these are usually extremely large (Borowitzka, 2005). One of us (X. G. Fei) has used a smaller prototype to culture conchospore-seeded nets of *P. yezoensis*. This raceway is a useful nursery system for nets seeded with *Porphyra*, and the system is inexpensive (ca. \$2500 US as compared to \$8000 for a manufactured tank), which makes it suitable for commercial use. The materials necessary to build the raceway are readily available, and the size of the raceway can be matched to any need. We established that a large number of nets (suitable for 667 m<sup>2</sup> of grow-out) could be accommodated in a small space. The nets were easily monitored in the raceway and convenient adjustments to the system were possible to maintain thallus growth and health. This is very difficult to do in the field, where weather can be a serious concern in terms of its effect on the germlings and access to the nets themselves. Constant flow in the raceway assured an even distribution of nutrients. Additionally, constant flow may encourage stronger attachment of germlings to the nets, and self-shading was reduced since the nets were pleated. All of the nets can be removed from the raceway and returned to the water within a few minutes by one person using the methodologies described here. Finally, the raceway can be isolated or run as an integrated part of a recirculating, land-based system. Several studies show that algae grown in effluent from finfish farms have high growth rates (Troell et al., 1997; Neori et al., 1998; Viera et al., (2006). When the raceway is incorporated into land-based aquaculture, the temperature and light can be

adjusted to maximize uptake of N by *P. umbilicalis* (Kraemer et al., 2004; Carmona et al., 2006). Lastly, the nets can be desiccated, as needed, to control epiphytic growth (Sahoo and Yarish, 2005).

This study demonstrates the feasibility of neutral spore-seeding of nori nets with a native Maine species of *Porphyra* and points to some areas where further methodological improvements are needed for crop development. At the nursery stage, joining the raceway to partial make-up flow from land-based, cold-water, fish aquaculture (e.g. salmon, halibut) would eliminate the need for the addition of fertilizer while making it possible to maintain a lower temperature of the seawater in the raceway, even in summer, without the added expense of a chiller system. The methods in this study could be integrated with both land-based and nearshore aquaculture.

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