Final Report: "Pilot Production of Native *Porphyra* for Land and Sea-based IMTA" (Project No. 12-06)

Introduction:

Porphyra is a nutritious sea vegetable based upon its protein, mineral and vitamin (e.g., B_{12}, C) content (Blouin et al., 2006, 2011). *Pophyra umbilicalis* is harvested from wild intertidal populations for human food from Maine, the Canadian Maritimes, Ireland, the United Kingdom, and France, among other locales, and has potential for profitable development in aquaculture for both human and animal foods (Gantt et al., 2010; Blouin et al., 2011).

This MAIC-funded project had three objectives:

1. To establish a net-seeding facility for *P. umbilicalis* ("laver", "nori") at the University of Maine's Center for Cooperative Aquaculture Research (CCAR) that could seed nets and store them for Maine companies and fishermen wishing to engage in grow-out for profit.
2. To demonstrate that waste effluent from land-based aquaculture could serve as the nutrient supply for growing up *Porphyra* on nets in the CCAR raceway while providing bioremediation for land-based aquaculture.
3. To provide seeded nets, if available, to interested commercial growers in Maine for trial grow-outs.

This project succeeded in establishing *Porphyra umbilicalis* for land-based aquaculture, as described below. Nets were seeded from wild (1x) and cultured (2x) material, and some of these nets were placed in the CCAR raceway while others were suspended at a nearby mussel farm. The results of net-seeding and grow-out research demonstrated limitations (see below) in the existing CCAR infrastructure that must be improved to establish a true seeding facility for coastal aquaculture and suggests that more information is needed on where to place seeded nets for grow-out on the coast.

<u>Establishment of net-seeding facility and blade grow-out using fish waste effluent</u>

The culture facility required as a center-piece of a net-seeding facility was established at CCAR in January 2012 (Fig. 1). We used one of the environmental chambers (Bush 10ft x 10ft x 20ft) previously acquired by Brown, Brawley et al. through a Maine Technology Asset Fund award to CCAR for aquaculture infrastructure improvement. Four fiberglass translucent cylinders (KallWall, Solar Components, Manchester, NH, USA) at CCAR were put into service for growing up material for net-seeding. This material was produced at CCAR in the following 6 months using an initial batch of strain P.um.1 of *P. umbilicalis* that was seeded onto glass beads (400 µm in diameter) by Brawley in her Orono-based laboratory (Blouin et al., 2007). A tablespoon of glass beads with 1 mm or smaller *Porphyra umbilicalis* strain 1 (P.um.1) growing on them was placed in each of two cylinders filled with UV-irradiated Taunton Bay seawater that was filtered with 5 µm and 1 µm filters

(Microvantage, Shelco Filters, Middletown, CT, USA) and also sterilized by CCAR's standard bleach/ sodium thiosulfate neutralization procedure. Cylinders were filled to 90-120 L volume with seawater. The West-McBride formulation of Provasoli's Enrichment was added to each K cylinder at 10 mL/L of seawater. This WES-PES enrichment includes inorganic minerals required for algal growth; the recipe's vitamins were omitted because the P.um.1 cultures were not axenic. A media lab was established at CCAR for the production of macroalgal culture media for a variety of species, and all WES-PES was eventually prepared on site. Light levels, provided by a bank of high output T12 fluorescent tubes (Phillips F96T12/841/HO) were measured across the cylinders with a range of 50-130 µmol photons/m^2/s. Cultures were bubbled vigorously from an air line entering at the bottom of the cylinder, which caused nori blades to tumble from the top to bottom of the cylinders. Initial photoperiod was 12: 12 (L:D), and the chamber temperature was set at 12° C.

Three weeks after starting the cultures from germinated spores on glass beads, material in tumble culture in the Kalwall cylinders had grown into small blades (0.5-1.5 cm) that had a deep red color. Two weeks after redistributing material evenly (40 g damp wt/ cylinder), these small blades had grown to 5.0-7.8 cm from holdfast to tip of blade and to 142 g damp wt in Kalwall cylinder 1 and 122 g damp wt in K cylinder 2 (increases of 305-355%). Thereafter, nori blades at a total 70-90 g damp wt were maintained in each K cylinder, which were expanded to a total of 4 Kalwall cylinders in the nursery seeding room. Large blades were produced (Figs. 2-3).

Seawater was exchanged wholly or by 25%-50% increments in K cylinders at 2-3 week intervals for the project period. For the first half of the project, WES-PES was added to the cultures at the recommended dose (see above) when seawater was changed or if blades began to turn green, a sign of N deficiency. For the last 3 months of the project period (i.e. April 24th onwards), we experimented with addition of only nitrate and phosphate at WES-PES concentrations to cylinder cultures, every other week to maintain color and growth in the environmental chamber with full seawater changes at ca. 3 week intervals. This was successful.

Cultures appeared free of contaminants in the first month of culture, but therafter, and especially after 2 months (early March) as warming occurred outside, small diatoms began to appear on the transparent plastic bag covering the Kalwall cylinders (i.e. the inner splashed area of the cover) and in an upper rim (air/water interface) of the cylinders. Significantly, maintaining about 80 g damp wt/Kalwall cylinder kept diatoms from fouling the blades or the rest of the Kalwall cylinder wall: This biomass of *Porphyra* out-competed the diatoms for nutrients and grew rapidly in the 90-120 L seawater volume and containers used. From May onwards, we experienced serious ciliate contamination in the cultures, and these were not eliminated, resulting in frayed edges as ciliates grazed blade edges. Both of these problems represent difficulties for long-term seeding facility work and are attributable to: 1) small cells that were not killed by UV or removed by filtration

during partial water exchanges and 2) possibly spread in the air from the rest of CCAR and its proximity to Taunton Bay.

On April, 3 additional Kalwall cylinders were set up in the CCAR greenhouse and fed from raceway effluent, which allowed blades to be grown up without fertilizing them artificially (Fig. 2). The required N and P nutrients came from excreted sea urchin waste in the seawater circulating across the raceway (i.e., from CCAR land-based sea urchin system; circulating seawater at this time contained 0.13 ± 0.03 mg/L NH_4^+, 0.02 ± 0.02 mg/L NO_2^-, and 0.89 ± 0.32 mg/L NO_3^-, T= 11.5 ± 1.1^oC; Mean ± SD, n=6 weekly measurements from 5/3/2012-6/29/2012). We grew up 3.7 kg of *Porphrya* during the project, using excess material harvested bi-weekly (Fig. 3) over the amount retained in each K cylinder for net-seeding (see below), and establishment of additional K cylinders' cultures. This amount of material represents a small amount of the growing and harvest potential during the 6 month project, because we lacked additional K cylinders for grow-out of blades, and much of the material grown up was fed to urchins or just held in the raceway.

Accomplishments:

1. We demonstrated that large blades of native nori (*Porphyra umbilicalis*) could be grown up in tumble culture without a change in morphology (e.g. curling), disease, or unintended losses of biomass to spore production.
2. Because many invertebrates can benefit from nori in their diets (e.g., mycosporine-like amino acid concentration, high protein), this project makes it immediately possible for a land-based aquaculture operation, such as sea urchin projects underway at CCAR, to grow nori as a primary or secondary food source and to do so inexpensively using animal waste excreted to the recirculating seawater as the nutrient supply. This was demonstrated to maintain blade cultures in this project. The K cylinders we used minimize footprint, although they are expensive.

Future Needs/Problems Identified:

1. It is important to establish sea vegetable seeding facilities under the simplest conditions possible, but the contamination problems that result from maintaining stocks in the same room where seeding and mass grow-out are occurring will always result in contamination of stocks. In both China and Japan, stock cultures are maintained under considerably more controlled and clean conditions than are currently possible at CCAR within existing infrastructure/available funding.
2. Some of the techniques we used, in discussion with Maine Coast Sea Vegetables, might make the crop produced ineligible for organic labeling (i.e., use of chemical inorganic nutrients in WES-PES), whether nori was grown-up in land-based or sea-based aquaculture farms. A possible way around this would be to sterilize filtered urchin/fish effluent to use in starter cultures, which should be considered in future work, as well as determining the evolving organic labeling requirements precisely.

Net-seeding and outplant to growers

We seeded three nets with *P. umbilicalis* neutral spores (Table 1, Figs. 4-6). These nets were purchased from China and included the Japanese proprietary, standard strand developed for nori culture in the 1960s (the industry standard).

The first seeding was done with wild *Porphyra umbilicalis* blades collected from the Maine shore (Fig. 4); the second and third seedings were done with spores released from nursery blades that had a 2-3 mm reddish margin with spores (Fig. 6). Spores were obtained per established methods (Blouin et al., 2007) and poured over folded nets, to which filtered seawater was added after 30 minutes (Blouin et al., 2007). The spore density was about half of that needed, so this process was repeated with the same nets 3 days later (Table 1). These nets were then left undisturbed in seawater in the seeding trays (rectangular, shallow tubs from Home Depot) for 3 days and then cultured in one of the Kalwall cylinders until small blades were visible. Three nets were transferred to the CCAR raceway and became fouled with diatoms quickly in this environment. The other net continued to develop blades and was eventually transplanted to a mussel farm, where it also was fouled by diatoms. Transferred back to the CCAR raceway for the summer, it has recently been returned to the field, where the farmer has been able to raise and dry the net every few days and small blades were appearing in December.

Spores were obtained from seed-stock of Pum1 grown onsite by similar procedures but although blades bore spores, the density released was lower than needed, resulting in a lower density on nets. However, these nets did produce useable densities of blades and were cultured in the Kalwall cylinders until small blades visibly covered the net. At that time, 2 nets were dried and then wrapped in plastic and stored in a freezer for x months before being outplanted to a shellfish farm. Outplanted nets became fouled with diatoms, whether stored in the freezer or not, but we demonstrated that nets could be stored successfully by taking some nets out of storage and growing them up in a Kalwall cylinder while outplanting others.

To attempt to increase spore release for the third seeding (the second from seedstock grown on-site), blades were scored with razor blades to make regular incisions about 1 cm deep at 3-4 cm intervals at the blade edge. This was designed to simulate amphipod grazing of field blades, which may increase spore release from blades by exposing sporangia in scalloped pockets on margins of field blades. The photoperiod was also altered to 10 h L: 14 h D prior to the last seeding, to see if more spores would be produced on seed-stock blades in the nursery facility under the natural photoperiod present when the greatest spore production occurs in the field. Despite these measures, spore release from the seedstock was similar to the second seeding experiment.

Two nets from both the second and third seeding were frozen for several months and re-submerged to test the practice of keeping seeded nets frozen as a means of storage for the industry. Nets were air dried on racks for 30 minutes and individually folded and placed into 3mil black plastic contractor trash bags. Each net was wrapped in its own bag, and then placed together inside another bag and stored in a deep freezer. These nets were removed on 10/15/12, and one net from each seeding was re-submerged in 90L 10°C UV sterilized filtered natural seawater in aerated Kalwall cylinders. The other two nets were given to a mussel farmer and transported in coolers to Walpole, ME, where they were stretched on frames and planted out, one hung underneath a mussel raft at 1 meter (3rd seeding), and one placed on 12" metal poles in the intertidal zone (2nd seeding). The net that hung underneath the mussel raft was periodically raised out of the water for a drying period of several hours approximately every 3-4 days. The net placed in the intertidal zone was covered in fine silt and no fronds were observed after 36 days. The net was relocated and hung off a dock for further observation. The net that was hung under the mussel raft did show 1-2mm fronds after 36 days, and continues to be under observation. The frozen nets that were re-submerged and cultured in the lab for 36 days showed good growth of fronds, with no contamination. The freezing step may accelerate development and reduce contamination stress that may be a problem in the nursery phase. These nets were cultured in tubes in the nursery for 36 days, then hung below the mussel raft for observation on 11/20/12.

Table 1. Summary of seeding experiments and outcomes.

Seeding date	3/7/12 (wild)	5/14/2012 (P.um.1)	6/10/12 (P.um.1)
Number of nets	4	6	6
Spore density	1.6 x10^5 spores/mL; 4 x 10^4 spores/mL (2 d later)	1.0 x 10^3 spores/mL	1.1 x 10^2 spores/mL
Blades 2-3 mm present on net	4/17/12	11/18/12 (after freezing—resubmerged on 10/15/12)	11/10/12 (after freezing—resubmerged on 10/15/12)
Nets left at CCAR	1 in cylinder; 3 in raceway	All grown for 1 month at CCAR; 2 dried and stored in freezer (6/6/12)	All grown for 1 month at CCAR; 2 dried and stored in freezer (7/10/12)
Outcome	cylinder=excellent growth, many blades; raceway=all nets heavily fouled by diatoms.	Frozen net cultured in cylinder, visible fronds by 11/18/12, outplanted at	Frozen net cultured in cylinder, visible fronds by 11/18/12, outplanted at

		mussel lease site on 11/20/12	mussel lease site on 11/20/12
Nets outplanted and outcome	1 (from tank) to mussel farm; heavily diatom fouled; moved to CCAR raceway for summer; now back in sea (no report), 3 placed in raceway at CCAR, all heavily fouled and discarded	Net from freezer stretched on wooden frame and placed in intertidal zone—no growth, net covered with fine silt, probably smothering growth. Net relocated to dock 11/20/2012 for continued observation	Net from freezer stretched on wooden frame and hung at 1 m under mussel raft with periodic drying, developing fronds visible by 11/20/12
Effect of storage of nets in freezer	n/a	Growth observed on nets after storage	Growth observed on nets after storage

Spores released from blades growing in tumble cultures in the seeding facility attached to the Kalwall cylinder's plastic walls near the top of the cylinders (Fig. 5: air/water interface). By April 9, some of these were 1 cm long and deep red in color. This allowed us to use these and smaller blades as "seed" for the culture facility; thus, this project found that the culture facility can be maintained indefinitely without going through a seeding step using spores on glass beads. The concentration of germinated spores at the air-seawater interface was unexpected, and it offers a possible strategy for seeding nets without a deliberate spore release step, although this would require a different type of container (square or rectangular tub with nets suspended at surface for a few days).

We also tested other available synthetic and natural twine (nylon and polypropylene, 4 mm diameter; cotton and polyester, 2 mm diameter; flat nylon braid, 2 mm; polypro, nylon and/or polyester, 2 mm diameter; all from Home Depot twine and rope stocks) as an alternative to standard nori net material from Asia in an ancillary experiment with tetrasporophytic dulse blades (Fig. 7). After 1 week of submersion of replicate pieces of each type of twine, spores were found only on the nori net pieces.

Accomplishments:

1. Seedstock was established at CCAR and maintained through 3 generations from spores that initially settled and germinated at the air-seawater interface of Kalwall cylinders with tumbled blades.
2. To supply growers, nets need to be produced before they are needed on order; we determined that this can be done by storing seeded nets, after

drying them for an hour, in heavy plastic bags in a walk-in freezer. These nets can be transferred to the field for grow-out.

Future Needs/Problems Identified:

1. The seedstock blades did not release spores in the concentrations needed. Thus, developing a good seeding technique needs urgent attention. The problem could be chamber photoperiod, constant immersion of blades (vs a diel cycle of aerial exposure) in tumble culture and/or other factors. Having only one chamber in which to do the work limited our ability to impose different environmental conditions on the blades during the MAIC-funded project to solve this problem, but its solution is likely to be simple given adequate facilities.
2. The major problems pertaining to continued development of this potential crop are infrastructural at the levels of personnel for field work (nets became fouled because no one was tending them; in Asia, nori nets are raised out of the water for several hours every few days to kill diatoms, because blades are resistant to desiccation) and seeding and growing facilities at CCAR. Nori needs strong circulation, temperature control, and natural light (e.g., in a raceway, Kalwall cylinder, or series of shallow tubs), seeding facilities that allow nets to be dipped into spore solution are required (e.g., on a carousel wheel) and nets must be outplanted quickly or dried periodically to prevent diatom growth.
3. Putting nets into the sea when nutrients are high and natural diatom blooms are occurring is poor husbandry. Both grazing of blades by small invertebrates and fouling of nets by diatoms may be problems. Dense seeding may compensate if nets are raised for drying in the field.

Presentations:

Redmond, S, Morse D, Brawley S, Brown N, Dobbins P, Eddy S, Erhart S, Fischer P, Larrabee J, Levesque T, Moretti M, Newell C, Olsen B, Olsen B, Olson T, Young E. 2012. Development of sea vegetable culture technologies in Maine In: Proceedings of the Northeast Aquaculture Conference and Exposition: Dec 12-15, 2012; Groton, CT.

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Blouin, N. B., X. G. Fei, J. Peng, C. Yarish, and S. H. Brawley. 2007. Seeding nets with neutral spores of the red alga *Porphyra umbilicalis* (L.) Kützing for use in integrated multi-trophic aquaculture (IMTA). *Aquaculture* 270, 77-91.

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life histories in a harsh environment. *P. umbilicalis*, the genomics' project. In: J. Seckbach & D. Chapman (eds), *Red Algae in the Genomic Age* (v. 13, Cellular Origins, Life in Extreme Habitats and Astrobiology), Springer, pp 129-148.

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Figs. 1: Enviromental chamber with Kalwall cylinders (4) used for growing sea vegetables: principally nori, was established at CCAR as a principal goal of the MAIC project. Fig. 1A shows all cylinders with tumbled nori cultures (grown-up from spores at CCAR). Fig. 1B shows dulse (facing camera) with other cylinders (behind) full of nori.

Fig. 1A

Fig. 1B

Fig. 2A. Greenhouse raceway with attached nori tumble cultures in translucent cylinders.

Fig. 2B. Greenhouse raceway with attached nori tumble cultures in translucent cylinders.

Fig. 3 A weekly harvest from one Kallwall; blades grew larger than blades found in the field, and a few were 23 cm x 30.5 cm (9” x 12”); a representative herbarium sheet will be deposited to the University of Maine’s herbarium.

Fig. 4. Net seeded with spores from field material covered with small blades (top, left) became heavily fouled with diatoms at mussel farm (top, right) but some blades had survived and grown to small size.

Fig. 5. Neutral spores released from seed-stock (P.um.1) blades in tumble culture attached and germinated at air-water interface in cylinders.

Fig. 6. Margin of seed-stock blades developed a reddish zone with spores (top, middle). Net seeded with spores from seedstock crop; note small blades.

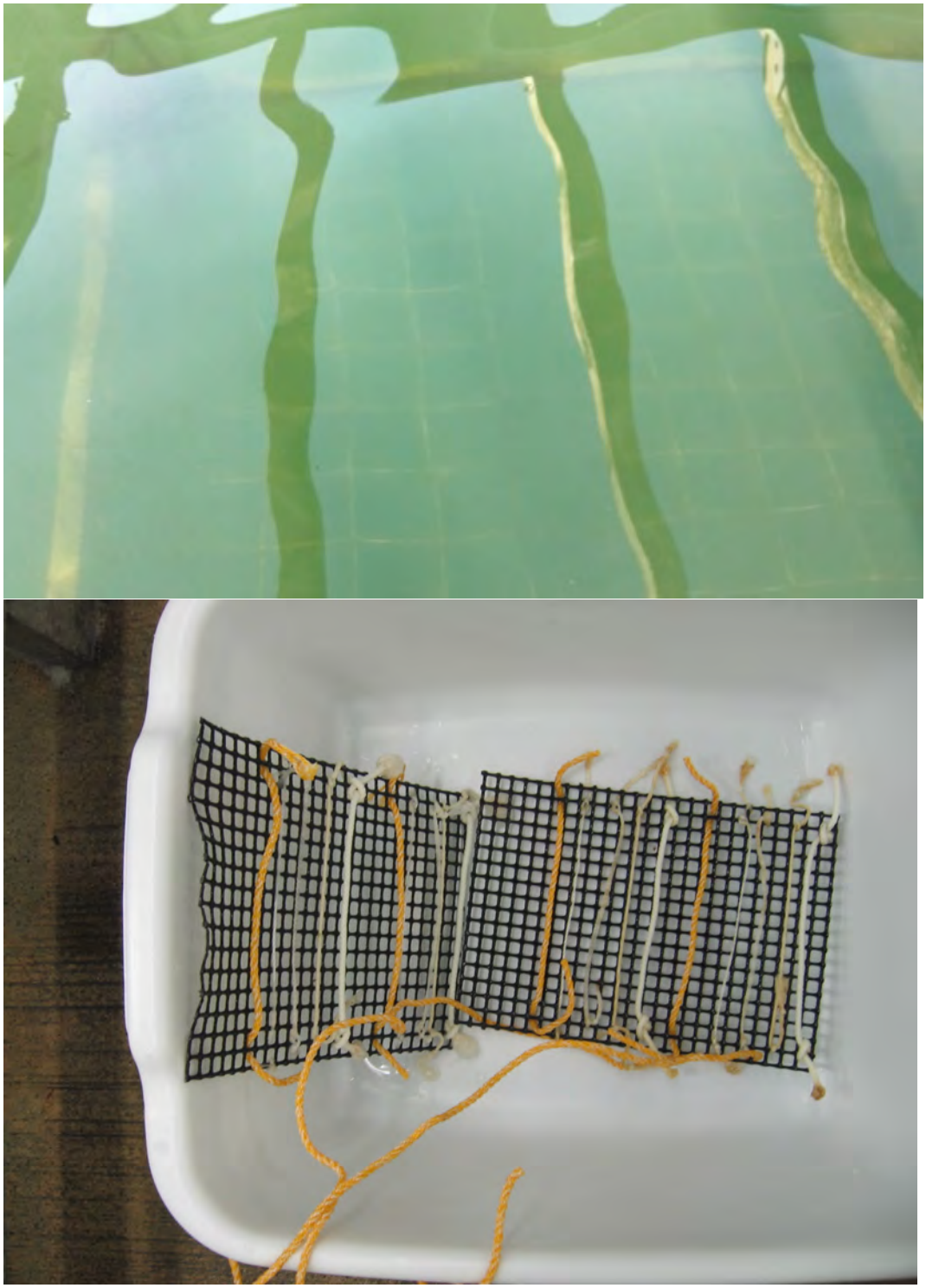

Fig. 7. Top: Nori net (top) suspended on wooden frame in shellfish farm; outplanted directly from frozen storage at CCAR in November 2012, and small blades were visible after a month, with this net dried every 2-3 days. Bottom: The design used for comparing string types for sea vegetable aquaculture (in this case, we tried various types [see report] in dulse cultures in one Kallwall).