TERMINATION REPORT - PART I

PROJECT CODE: 03-14

SUBCONTRACT/ACCOUNT NO: 556805 (Grant # 99-38500-7885)
556908 (Grant # 00-38500-8990)

PROJECT TITLE: **Salmon Hatchery Effluent Management Utilizing Integrated Polyculture Technologies**

REPORTING PERIOD: January 1, 2004 – August 1, 2006

FUNDING LEVEL: $ 150,000

PARTICIPANTS: Ira Levine, Donald Cheney, Rebecca Lebrun, Tony Legee, Bobbi Cooke, Hitoshi Kito, Dongdong Kong, Zhing Pei

REASONS FOR TERMINATION:

Indicated objectives completed

PROJECT OBJECTIVES:

1. Development of a "Zero-salinity" or freshwater tolerant, fast-growing strains of *Porphyra yezoensis*.

2. Determination of optimal *Porphyra yezoensis* cultivation strategies for N and P removal.

3. Identification of molecular aspects of salinity tolerance.

4. Technical transfer of project results.

ANTICIPATED BENEFITS:

Benefits include the newly developed euryhaline cultivars capable of producing r-phycoerythrin in freshwater aquaculture systems. Determination of N and P uptake dynamics, and the understanding of the molecular aspects of salinity balance in *Porphyra*. The competitive advantage of integrated polyculture will assist the land-based finfish industry in competing with global commercial interests, reduction of finfish effluents, and development of a second cash crop.

PRINCIPAL ACCOMPLISHMENTS:

1. Development of *Porphyra yezoensis* cultivar maintaining growth rates of 10 -15% per day in salinities as low as 8ppt. Additionally, cultured *Porphyra yezoensis* grown in 0.125 ppt seawater supplemented with divalent cations sustained growth rates of 3% per day.

2. Osmoregulated *Porphyra yezoensis* (supplemented with divalent ion cocktail) absorbed 16.6 – 23% of the freshwater trout effluent available nitrogen-nitrate with and without nutrient enrichment.

3. The isolation and sequencing of the putative *Porphyra* CAS.

4. One state, one regional and two international presentations of the grant's results. Additionally, one peer reviewed article has been published.

IMPACTS:

As the salmon aquaculture industry significantly reduces production and the number of hatcheries in the State of Maine, the challenge of effluent remediation by the originally intended end-user of this research becomes moot. The development of osmoregulated algal polyculture systems will become a reality when higher growth rates are achieved or when brackish water aquaculture systems are identified.

RECOMMENDED FOLLOW-UP ACTIVITIES:

Continued funding for CaS research and the development of osmoregulated algal culture systems are necessary to advance this concept further.

SUPPORT:

YEAR	NRAC-USDA FUNDING	OTHER SUPPORT					TOTAL SUPPORT
		UNIVERSITY	INDUSTRY	OTHER FEDERAL	OTHER	TOTAL	
2004	75000	15000					$ 90,000
2005	75000	18645					$ 93,645

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

Papers Presented

Levine, I., D. Cheney. 2005. *Porphyra* from sea to freshwater; osmoregulation in marine algae. 44th Annual Meeting Northeast Algal Society. Rockport, Maine. Book of Abstracts: 19.

Liu, Y.C., T Hogan, A. Cary, A. Silvestro, L. Graham, D. Cheney, and I. Levine. 2005. Investigations of the tolerance ability of *Porphyra* to survive extreme environmental stresses. 44th Annual Meeting Northeast Algal Society. Rockport, Maine. Book of Abstracts: 21.

Levine, I., A. Legee, Z. Pei, and D. Kong. 2006. *Porphyra* osmoregulation and the isolation of a Ca^{2+} sensing receptor. 54th Annual Meeting British Phycological Society. Plymouth, UK. Book of Abstracts: 17.

Levine, I., A. Legee, Z. Pei, D. Kong, D. Cheney, and T. Hogan. 2006. Osmoregulation in marine algae and the bioremediation of freshwater effluents. International Conference on Applied Phycology; Algae in Biotechnology and Environment. Delhi, India: Book of Abstracts: 8-9.

Publications

Levine, I. 2006. Case study on *Porphyra* cultivation in Maine, USA and New Brunswick, Canada. In: Aquaculture Compendium, Online at www.cabicompendium.org/ac. Wallingford, UK: CAB International. pp 1-13.

Termination Report - PART II

In the northeastern United States, Atlantic salmon and other finfish (trout, tilapia, halibut, cod and haddock) are the dominant aquacultured species with > 20 finfish hatcheries whose ability to meet EPA's new discharge standards is a matter of concern. The development of a practical integrated seaweed: finfish integrated aquaculture system would bioremediate hatchery effluents while producing a second commercial crop. Examples of valuable byproducts found in *Porphyra yezoensis* include: antioxidant carotenoid pigments such as beta-carotene (0.705 mg/g DW) and lutein (1.339 mg/g DW), long-chained polyunsaturated omega-3 fatty acids such as eicosapentaenoic acid (EPA; 6-12 mg per g DW), various vitamins (C, E and B12), and the pigment r-phycoerythrin C, which is utilized as a fluorescent “tag” for immunofluorescent studies.

One advantage of modifying marine algae, e.g. *Porphyra* spp. to bioremediate effluents from a freshwater fish hatchery is the ability to absorb ammonia and nitrate simultaneously at high rates, in contrast with phytoplankton and many vascular plants which often stop using nitrate as ammonia levels increase.

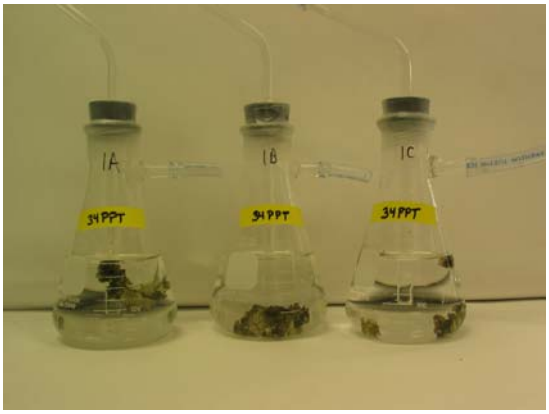
Calcium ions have been demonstrated to exert key regulatory effects on algae when exposed to low salinities. Previous reports suggest that: 1) calcium is a key element in determining algal survival at low salinities and 2) that genetic isolates of various seaweeds may possess alterations in distinct genes including Calcium Receptors (CaR) that provide them with the ability to adapt to the shifting salinities. CaRs have been shown to be the “master controller” of divalent calcium homeostasis in humans and salinity sensors in flowering plants, aquatic organisms, and green algae. Preliminary efforts have suggested similar molecules in *Porphyra*.

The development of a land-based, coastal independent system, utilizing low salinity-tolerant (via classical and emerging proprietary methodologies) strains of *Porphyra* was the focus of both cultural and molecular investigations funded through the USDA NRAC Grant 03-14.

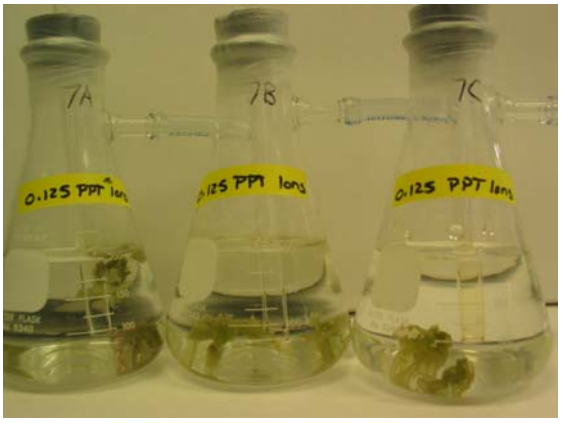
Objective 1:

Initial efforts have yielded sporophytic and gametophytic strains of *Porphyra yezoensis* that maintain 10 -15% per day growth rates in salinities as low as 8ppt. Our team has successfully cultured *Porphyra yezoensis* in 0.125 ppt seawater supplemented with divalent cations achieving limited growth rates of 3 % per day in 16 °C at reduced light levels. PI increased the scope of the year one research plan and included both the gametophyte (as originally described in the grant proposal) and the sporophyte or conchocelis form of the plant life history (Addendum I) in the low salinity experimentation.

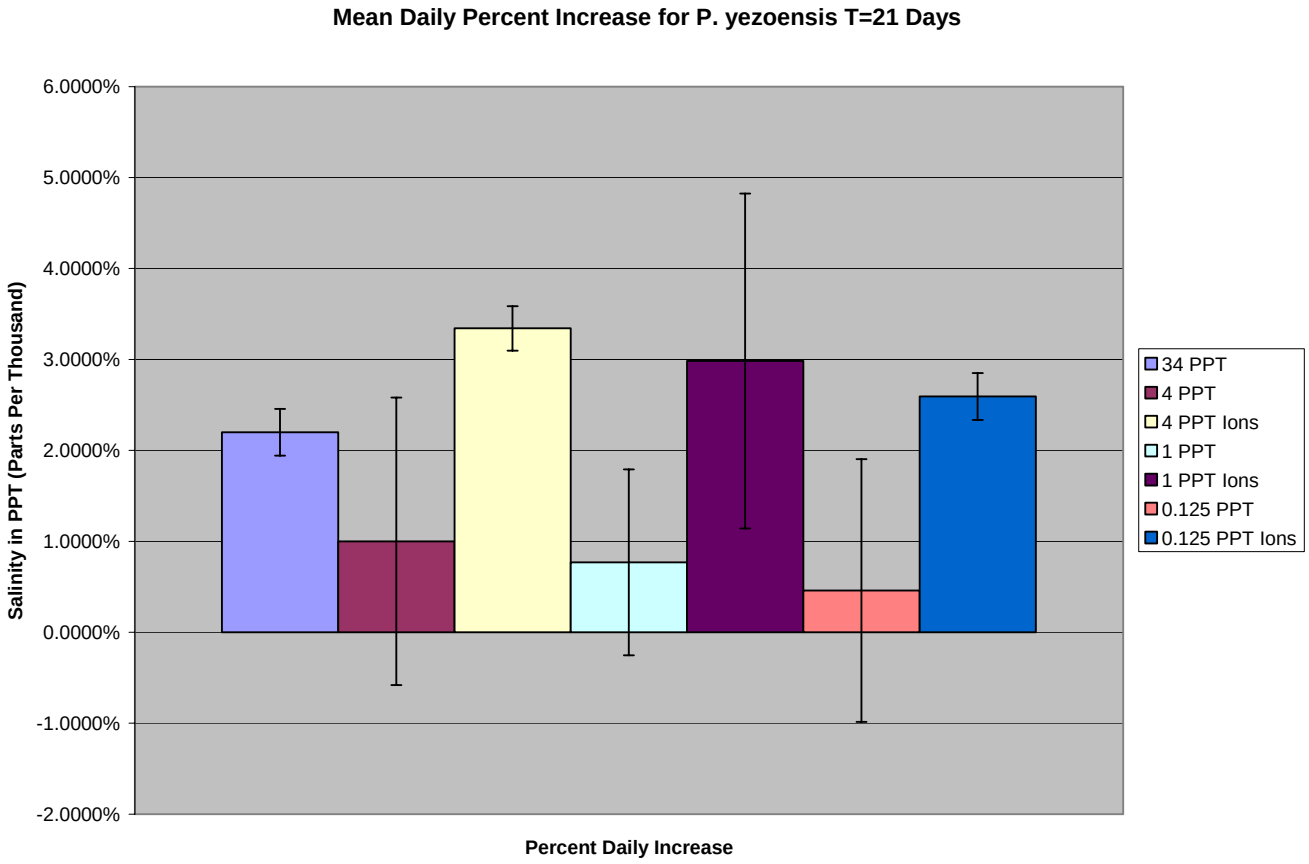
The development of a freshwater *Porphyra* cultivar, the singular goal of year one of this two-year research effort, has been achieved. Thalli grew at a greater rate although not significantly greater in ion-supplemented freshwater as compared to the control of full strength seawater.



Full Strength Seawater (32 ppt) T= 21 days



Freshwater (0.12 ppt) T = 21 days



The cultivation research in months 13-18 have been significantly hindered by the cross contamination of our low salinity cultures by an unidentified cyanobacteria which increased its growth rates as a function of the decrease in salinity.

Objective 2:

Osmoregulated *Porphyra* nitrogen uptake dynamics are independent of salinity variation (0-34ppt). Osmoregulated *Porphyra yezoensis* (supplemented with divalent ion cocktail) absorbed 16.6 – 23% of the freshwater trout effluent available nitrogen-nitrate with and without nutrient enrichment, respectively as compared to 17% absorption of the full seawater control over the course of the 7-day experiment (Figure 1). The highest rate (m = -0.023) of nitrate uptake was realized in the 0ppt fish effluent with ion supplements and ESS (Figure 2); uptake was approximately 35% more rapid then that of ion supplemented fish effluent without extra nutrients added.

Over the course of the 7-day experiment, nitrate absorption was evident in all, but the non-osmoregulated, 0 ppt fish effluent without supplemental ion treatments. The non-osmoregulated thalli treatment increased in nitrate levels (+ 29%) possibly due to tissue deterioration and pigment loss observed after 48 hours. Observations of both osmoregulated treatments (with and without added ESS) and the full strength seawater control indicated substantial tissue integrity and pigment retention. Comparison of uptake rates between the osmoregulated thalli cultured in fish effluent without ESS nutrient supplementation and that of the full seawater control reveals nearly identical uptake rates (m = - 0.0087 and – 0.0092, respectively).

Figure 1. Osmoregulated *Porphyra yezoensis* Nitrate Uptake

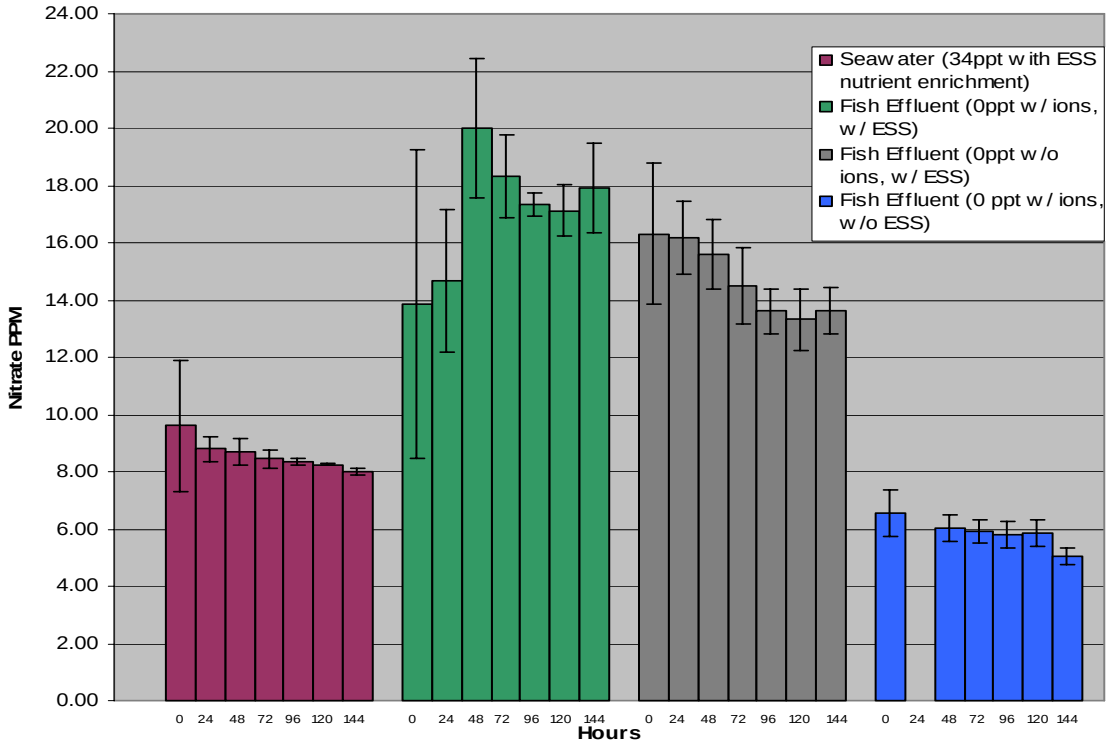
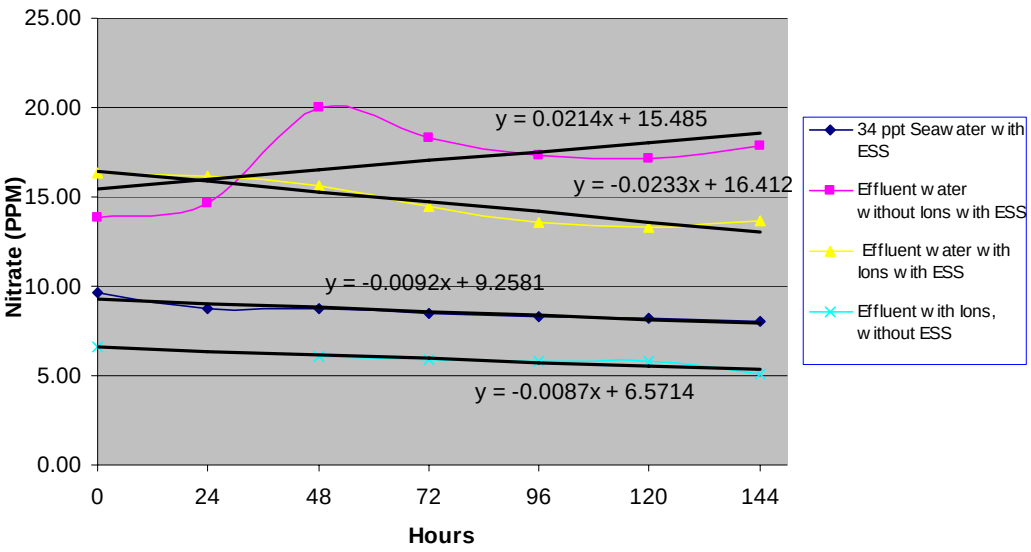


Figure 2. Nitrate Uptake Rates



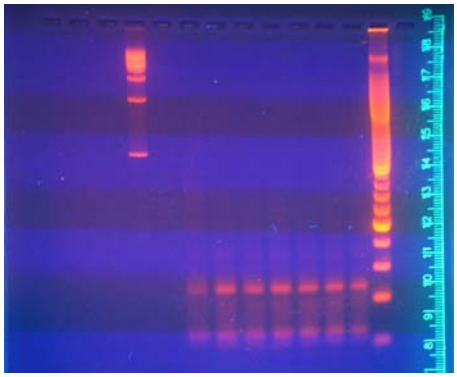
Objective two's research effort determined the ability of osmoregulated thalli to bioremediate fish effluents as part of a polyculture system. The data revealed nearly equal uptake rates between the control and the osmoregulated thalli. Thalli integrity and pigment concentrations were also observationally equal indicating a healthy system.

The highest rate of nitrate uptake was observed in the nutrient supplemented, osmoregulated treatment which would indicate the ability to bioremediate hyper accumulated fish effluents along with land-based, agricultural produced effluents.

Objective 3:

The development of a freshwater *Porphyra* cultivar, the singular goal of year one of the two-year research effort was achieved (see Year 1 Annual report dated November 19, 2004). As per the annual report the efforts to develop the molecular nature of salinity tolerances, the cloning of the CAS gene has made significant progress.

Results to date include the isolation and sequencing of the putative Porphyra CAS. Database analyses indicate homologues inOryza sativa, Arabidopsis thaliana, and Chlamydomonas reinhardtii. Future efforts include determination of Ca^{2+} binding capacity, and subcellular localization.



CAS gene cloned from *Porphyra yezoensis*

Part II -- Adendum I

Objectives

The main objectives of our work during the first six months of this project were to
1) Investigate the best methods for growing conchocelis of *Porphyra yezoensis*, 2) determine its salinity tolerance limits, and 3) initiate studies to produce a low-salinity tolerant strain that is faster growing than is being currently being tested.

Major Accomplishments to Date

1. **Establishment of conchocelis culture conditions**.

"Conchocelis" refers to the filamentous, diploid stage in the life cycle of a *Porphyra* species (see Figure 1). We are interested in learning how to grow conchocelis because of its very different, and potentially valuable, polyunsaturated fatty acid (PUFA) composition from that found in blades. In particular, previous work (Graham and Cheney, unpublished) has shown that the most abundant PUFA found in conchocelis of *Porphyra yezoensis* is arachidonic acid (AA), while that found in blades is eicosapentaenoic acid (EPA); both can play an important role in the early development and growth of fish and are of commercial value.

TERMINATION REPORT – Part III

PROJECT CODE: 03-14

SUBCONTRACT/ACCOUNT NO: 556805 (Grant # 99-38500-7885)
556908 (Gant #00-38500-8990)

PROJECT TITLE: Salmon Hatchery Effluent Management Utilizing Integrated Polyculture Technologies.

Although our laboratory has a great amount of experience growing the haploid blade stage of *Porphyra*, we and most labs have very little experience growing the conchocelis stage. Thus, one of our first goals was to determine the best culture conditions for growing conchocelis. However, before we could initiate conchocelis culture experiments, we had to have "clean" (i.e. contaminant free) culture material. The conchocelis material we had growing in our lab we discovered was contaminated with yeast and bacteria. We developed a simple "conchocelis decontaminating method" that kills these and other contaminants but does not harm the plants. The method consists treating the conchoecelis with a 1% solution of the commercial disinfectant Proviiodine Iodine Solution (which is a 10% iodine solution) for 60 seconds. This cleaning method has also worked on decontaminating a new conchocelis culture we received from Japan (see below).

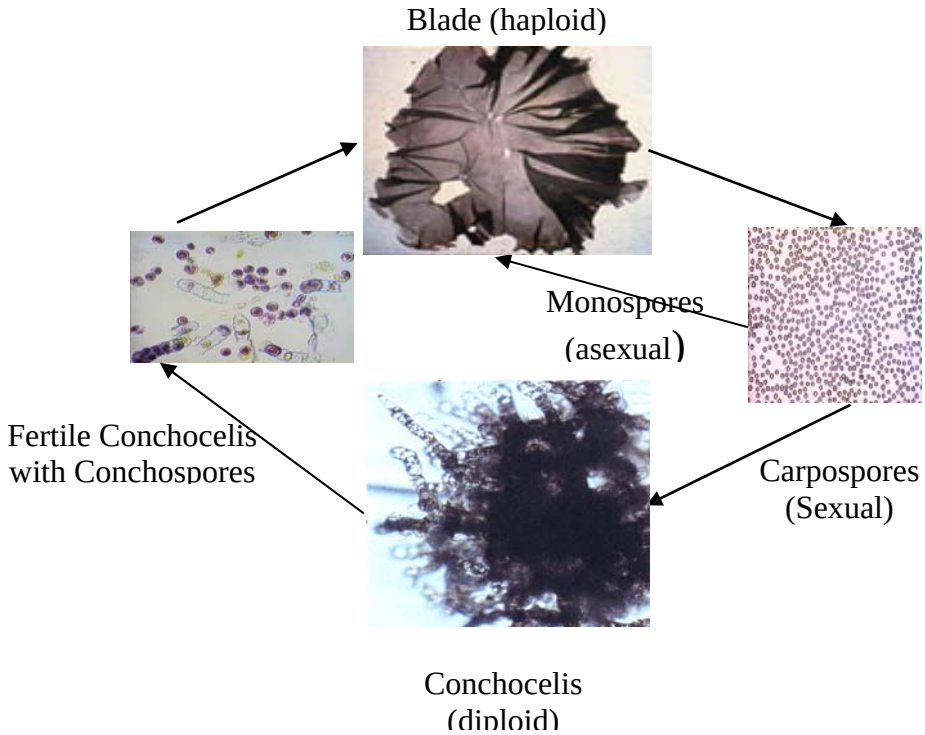


Fig. 1. Porphyra life cycle

Initial culture experiments were conducted to determine the best preliminary light and temperature conditions for growing conchocelis of two strains of *P. yezoensis* (strain #9-13, a patented strain produced by my lab over five years ago, and its parent strain #U-51) in wells. The conchocelis was subdivided into small clumps 1-2 mm in dia and grown one clump per well (see Figure 2) in a "24-well plate." Because the conchocelis in each well is too small and light to weigh, we measured growth over a 3 week period by estimating the change in clump size over time using methods similar to those used in the past to measure conchocelis growth (e.g. Waaland et al 1987; Varela-Alvarez et al 2004).

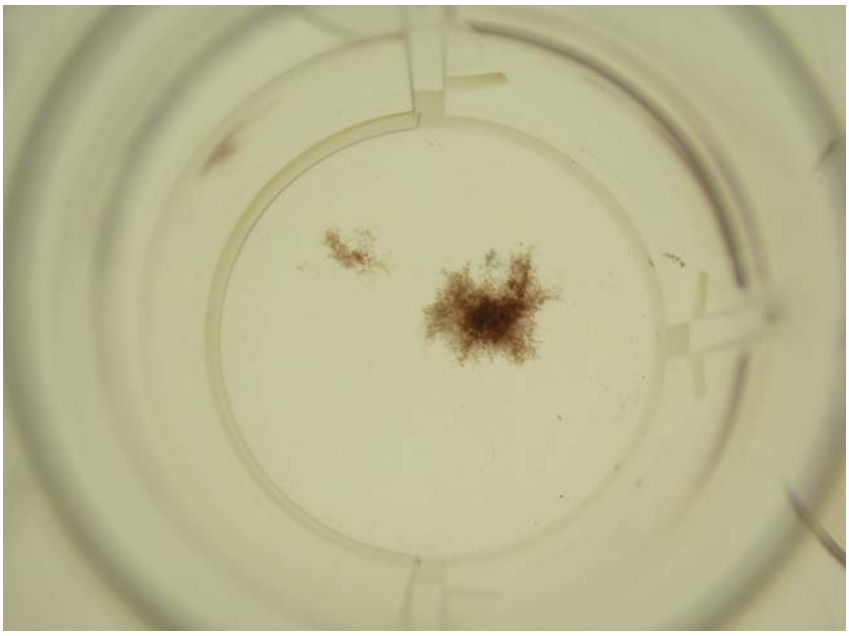


Figure 2. Conchocelis growing in a well of a “24-well plate”

Preliminary results include:

1. Both conchocelis strains could be quickly increased in biomass by subdividing it weekly and growing it in wells of a 24-well plate. After a clump was “subdivided”, we observed that the “daughter” clumps of conchocelis tend to "attach" or stick to the bottom of the well, a trait that could be useful for growing large amounts of conchocelis as a “mat” covering a surface.

2. Of the four culture conditions we initially tested (high and low temperature and light); the best growth was observed at the higher temperature (16^{o} vs 8^{o} C) and lower light level (ca 10 µEinstein vs. 35 µEinstein) conditions. Strain #9-13 grew faster than #U-51 (see Figure 3) at these conditions and was subsequently used for salinity tolerance experiments.

2. Initial testing of salinity tolerance.

To our knowledge, there is no information available on the salinity tolerance of any *Porphyra's* conchocelis. So far we have completed only one experiment to determine the salinity tolerance of strain #9-13 conchocelis. A second experiment is underway. In our initial experiment, we measured growth at three salinities full strength seawater (32 ppt), 20 ppt, and 8 ppt. These salinities were selected because of comparable growth data for the same salinities we had for blades. As can be seen in Figure 3, the growth rate we got at 20 ppt was almost as high as at full strength seawater (250% vs. 300% increase or 2.5X vs. 3X after three weeks), while that at 8 ppt was significantly less (150% or 1.5X after three weeks). Although these growth rates are less than what we found for blades, interestingly the pattern of salinity effects is similar to that for blades. That is, there appears to be very little difference between 32 and 20 ppt but a big difference between 32 and 8ppt. Thus, because conchocelis appears to be behaving similarly to blades, we would predict that the methods that Dr. Levine is using to grow blades at "0-salinity" should work with conchocelis. We will test this idea during the next phase of the project.

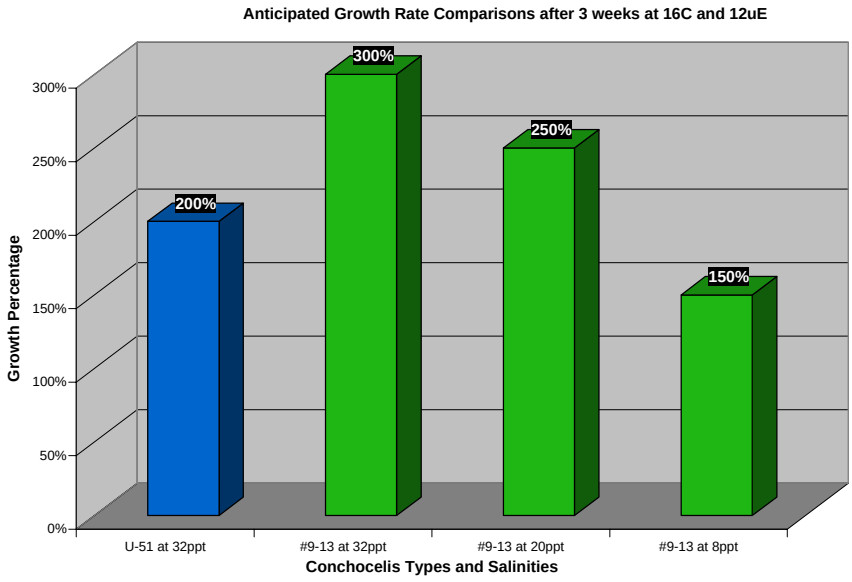


Figure 3. Growth Rate comparisons after 3 weeks at 16°C and 12 µE

3. Initial efforts to establish a "free-living" conchocelis culture system.

There are reports that *Porphyra* culturists in Japan and China are able to grow conchocelis at a fast rate in a so-called "free-living" culture system rather than a "standing" system such as in wells or the traditional oysters (Charles Yarish, personal communication). We have initiated studies to determine if we can grow strain #9-13 conchocelis in an aerated flask. So far, we have found that we can grow small fragments of conchocelis in bubblers, but only at low light levels. That is, fragments grown at higher light levels (i.e. above 12 µEinstein) become reproductive and start to produce blades, whereas conchocelis grown at light levels below 6 µEinstein remained non-reproductive. We think this may be a very promising approach and needs more experimentation.

4. Culture of new "fresh water" strains from Japan

We received two new *Porphyra* conchocelis cultures from Dr. Levine that are purported to be "fresh water" strains. These include : *P. tenera*, collected 4/9/04 from river mouth of Nagata river in Shimonoseki, and *P. katadae*, collected 2/17/04 from river mouth area of Kawatana river in Toyoura-gun, Japan. The *P. tenera* culture we received was contaminated with blue-green algae and had to be decontaminated using our "conchocelis-cleaning method" described above.