

10-11-2012

Gracilaria Culture Handbook for New England

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Recommended Citation

Yarish, Charles; Redmond, Sarah; and Kim, Jang K., "Gracilaria Culture Handbook for New England" (2012). *Wrack Lines*. Paper 72.
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New England *Gracilaria* Culture Handbook

System Implementation & Operation
For Production of Young Plants

**Project Title: Seaweed Aquaculture for Bioextraction of Nutrients from LIS and
Bronx River Estuary (Project # 24266**)**

Date: Oct. 12, 2012

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**Long Island Sound Futures Fund (LISFF) and
The National Fish & Wildlife Foundation Award Notification (Project # 24266)

This project was funded by the Long Island Sound Futures Fund (National Fish and Wildlife Foundation), under project title “Bioextraction of Nutrients from Long Island Sound” (1/3/2011-4/2/2012) to Dr. Charles Yarish, University of Connecticut. Additional support was provided by the Connecticut Sea Grant College Program (Project Number R/A-38), under the title, “Development of Seaweed Culture System Technologies to Support Integrated Multi-trophic Aquaculture and Sea Vegetable Aquaculture in New England Coastal Waters” (2/1/2010-1/31/2013). The principal investigator wishes to thank his co-PIs including Dr. George P. Kraemer (Purchase College, Purchase College), Mr. John Curtis (Bridgeport Regional Aquaculture and Science Technology School in Bridgeport, CT and to his UConn seaweed research team including Sarah Redmond and Dr. Jang K. Kim. We would also like to thank the many the UConn and Purchase College undergraduate students, BRASSTEC students and staff, and Rocking the Boat (Mr. Adam Green and Ms. Dawn Henning) for all their efforts logistical support for the Bronx River estuary and Long Island Sound sites.

Preface

The seaweeds are a diverse group of large marine macroalgae that are as important to the marine world as land plants are to our terrestrial world. Seaweeds were the precursors to land plants, and like land plants, they are critical primary producers, forming living links between the inorganic and the organic world, using photosynthesis to convert CO₂ and nutrients into living biomass. These primary producers support and encourage other marine life through the production of oxygen, contribution to marine food webs, and by providing structure and habitat for refuge, breeding, and nursery grounds for fish and invertebrates. Seaweeds are an important resource for humans. Coastal peoples have always utilized seaweeds for food, minerals, medicine, fertilizer and fodder, and these sea-plants continue to provide for an ever-increasing world population. A multi-billion dollar industry worldwide, seaweeds are consumed as nutritious sea vegetables, processed to extract valuable phycocolloids including agar, carrageenan, and alginate, and utilized in the agricultural industries for animal fodder or plant fertilizers.

Wild harvest still constitutes a significant portion of seaweed production, however, there is an ever increasing amount of seaweed production from aquaculture, principally in Asia (China, Korea and Japan) and South America (Chile). Seaweed aquaculture makes up about 24% of all aquaculture production (~19 million metric tons) with a value of ~US \$5.65 billion (FAO, 2012). Most of the cultured harvest is the kelps, *Saccharina japonica* and *Undaria pinnatifida*, the red algal carrageenophytes species including *Kappaphycus* and *Eucheuma*, the edible red algal species known as nori (including *Porphyra* and *Pyropia* species) and the red algal agarophyte species known as *Gracilaria*. China is the world's top producer of cultured seaweeds, though other countries in Asia (Japan, Korea, and the Philippines) and in Europe (France, Ireland, Norway, Scotland, and Spain) also grow seaweed. In North America, seaweed industries are mainly small wild-harvest cottage operations located along the East and West Coasts of Canada and the United States, however, there is a newly developed sugar kelp industry in the Gulf of Maine (Maine and New Brunswick, Canada).

As populations expand, culture of seaweeds will be important to supplement the wild resource. Seaweeds can be cultivated like land plants, in the sea on suspended lines, rafts, or nets, or on land in tank-based culture systems. A sustainable, low- impact process, seaweed culture can provide much needed employment and independence to rural coastal areas. The development of a seaweed aquaculture industry can also encourage development of other aquacultured species higher up in the food chain, since seaweeds are bioextractive organisms, taking up excess nutrients generated by other species, such as fish or shrimp. The integrated culture of fed aquaculture (fish and shrimp) with extractive aquaculture (seaweeds and shellfish) has been called 'Integrated Multi-Trophic Aquaculture', or IMTA. The IMTA concept is an ecologically

based model that couples an inorganic bioextractive organism (seaweed) with an organic bioextractive organism (shellfish) to balance the intensive culture of fed organisms (finfish and shrimp), in order to produce a more sustainable, cleaner, and diversified aquaculture system (Neori *et al.*, 2007). The development of new ecologically based, sustainable culture technologies will ensure future employment for coastal communities, healthier coastal ecosystems, and the protection of important wild populations.

There are many interesting seaweed species in the Northeast with great economic and environmental potential. There are economically important kelp species—*Alaria esculenta*, *Laminaria digitata*, and *Saccharina latissima*, which are all large brown algae. They are the largest of the North American seaweeds, some species exceeding 10 m in length. They are an excellent source of iodine and other trace minerals, as well as a source of alginate, a phycocolloid used in many different industries (Sahoo and Yarish, 2005). The native red seaweeds of interest in the Northeast include *Gracilaria tikvahiae*, *Chondrus crispus*, and *Porphyra/Pyropia* (hereafter referred to as *Porphyra*) species. *Gracilaria* species are cultivated at a large scale in some countries for food, as a feed for abalone, and for agar, an important phycocolloid in the food, medical, and microbiological industries. *Chondrus crispus* is cultured in land-based tank systems for sea vegetables and wild harvested for a variety of carrageenans, which are important in the food and consumer products industries as thickeners and stabilizers. *Porphyra* species are cultivated on nets in Asia and are pressed and dried into the valuable nori sheets that are an integral part of the Asian diet. In New England, the development of *Porphyra* cultivation in land-based recirculating IMTA systems has been a topic of particular interest (Yarish and Pereira, 2008; Pereira and Yarish, 2010). However, it will be the goal of this manual to introduce cultivation techniques for native red alga *Gracilaria tikvahiae* to the inquisitive aquaculturalists, as well as providing a resource to coastal managers. A video that compliments this Manual on *Gracilaria* cultivation may be found at <http://digitalcommons.uconn.edu/wracklines/71/>.

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Introduction

Worldwide demand for seafood has increased steadily over the years, while for the past 20 years the amount of seafood harvested from wild populations has remained constant (FAO, 2002, 2003, 2006, 2010, 2012; New 1999). The increased demand has been met entirely through marine aquaculture, which is a multi-billion dollar industry (FAO, 2010, 2012; Fig 1). The United States is a major consumer of marine aquaculture products, yet we grow only a small fraction of what we consume (FAO, 2010). A major obstacle to the growth of a U.S. aquaculture industry has been the need to find environmentally sustainable methods of farming fish and crustaceans. Two issues of particular concern are 1) nutrient loading of coastal waters from effluent generated by coastal and shore-based fish aquaculture operations, and 2) the depletion of wild fish stocks harvested for fishmeal used in marine finfish aquaculture diets.

One goal for the development of domestic sustainable aquaculture is to ensure that commercial aquaculture has minimal adverse effects on the environment. One way to achieve this goal is through development of improved methods of waste management for land-based and coastal/offshore aquaculture. Fish, through metabolic processes, excrete nitrogen, phosphorus and carbon dioxide (Beveridge 1987; Mugg, *et al.* 2000; Neori *et al.* 2004, 2007). Up to 49 kg nitrogen (N) and 7 kg phosphorus (P) can be released per ton of finfish produced per year (Chopin *et al.* 1999; Kautsky *et al.* 1996; Troell *et al.* 2003). In coastal waters, high levels of these nutrients can trigger harmful algal blooms and contribute to excessive growth of nuisance algae, which in turn have serious negative consequences on coastal ecosystems and the economy. These nutrients could instead be used to support the growth of economically important seaweeds (Neori *et al.* 2004; Chopin *et al.* 2008, Yarish & Pereira, 2008).

On a global basis, seaweed aquaculture represents 24% of world marine aquaculture production on a weight basis and \$5.7 billion U.S. dollars on a monetary basis (FAO 2012). Nearly all seaweed aquaculture occurs in China, Korea and Japan. North America has very few seaweed aquaculture operations. The most notable in the Northeast is Acadian Seaplants, LLC, in Charlesville & Dartmouth, Nova Scotia, CA, producing the red seaweed *Chondrus crispus* for export to the Japanese food market (Craigie and Shacklock, 1995, Craigie

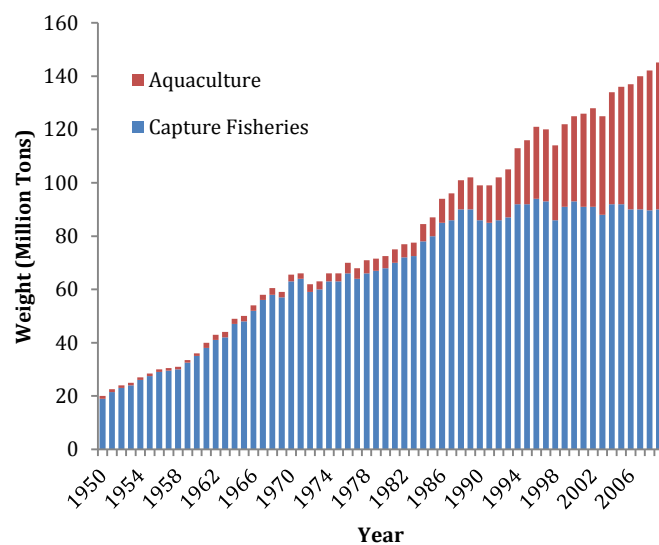


Figure 1. World Aquaculture Production and Capture Fisheries Data (FAO, 2012).

et al. 1999). The primary commercial use of seaweed is as human food, either as sea vegetables or functional foods (Cordero, 2006; Nisizawa, 1987, 2006; Smit, 2004; Teas, 2006). Seaweeds are also used as a source of colloids for the food and cosmetic industries, as an ingredient in pharmaceuticals and nutraceuticals, as a supplement in livestock feed and as a soil amendment in agriculture (Yarish and Pereira, 2008; Neori, 2008; Braden *et al.* 2007; Saker *et al.* 2003; Turner *et al.* 2002; Pereira and Yarish, 2010, Pereira *et al.*, 2012; Holdt and Kraan, 2011; Craigie, 2011; Browdy *et al.* 2012). It has been used as a food source for abalone and urchin aquaculture and is currently being investigated as a replacement for fishmeal in finfish diets (Neori *et al.* 2004; Neori *et al.* 2007; Robertson-Anderson *et al.* 2008; Francis *et al.* 2008, Walker *et al.* 2009). In the 1980s, there was significant interest in seaweeds as a biomass source for methane production (Flowers and Bird, 1984; Gao and McKinley 1994), and there is current renewed interest in seaweed as a biofuel source for ethanol and methanol production (Horn *et al.* 2000; Matsui *et al.* 2006; Yokoyama *et al.* 2007). However, the potential of most seaweeds as a feedstock for biodiesel is low, as the lipid content generally tends to below 5% dry weight except for some species of *Porphyra/Pyropia*, *Codium* and *Hypnea* (Sanchez-Machado *et al.* 2004; Pereira and Yarish, 2010). There are a number of potential applications for habitat restoration (Carney *et al.* 2005), have been proposed as large-scale carbon sinks, as a method of removing heavy metals from marine environments (Davis *et al.*, 2003), and even as a way to detoxify and remove TNT from seawater (Cruz-Urbe *et al.* 2007).

Seaweeds have been successfully incorporated into a number of demonstration and pilot-scale IMTA and nutrient bioextraction systems. A pilot-scale coastal IMTA project in New Brunswick, Canada uses kelp (*Saccharina latissima* and *Alaria esculenta*) and mussels (*Mytilus edulis*) as the extractive components in close proximity to salmon (*Salmo salar*) net pens (Chopin *et al.* 2008). In Portugal, Matos *et al.* (2006) demonstrated the effectiveness of three red seaweeds, *Palmaria palmata*, *Gracilaria bursa-pastoris* and *Chondrus crispus* in removing nutrients from the effluent of tank-based production of turbot (*Scophthalmus maximus*) and sea bass (*Dicentrarchus labrax*). In Israel, Neori (1996) and Neori *et al.* (1996, 1999, 2000, 2003, 2004, 2007, 2008) have developed small commercial scale IMTA systems incorporating gilthead seabream (*Sparus aurata*), the green seaweed *Ulva lactuca*, abalone and sea urchins. In South Africa, kelp (*Ecklonia maxima*) grown in the effluent of abalone aquaculture tanks was fed back to the abalone. Nutrient load in the effluent was significantly reduced and more of the exogenous nutrients were converted to abalone biomass (Bolton *et al.* 2006; Troell *et al.* 2006; Robertson-Andersson, 2008; Francis *et al.* 2008). The seaweed in these systems can be used for human food, as a protein source in finfish aquaculture diets, as a source of pharmaceuticals, phycocolloids and other biochemicals, or as a carbon source for biofuel production (Horn *et al.* 2000; Chopin and Sawhney, 2009; Chopin *et al.* 2008; Buschmann *et al.* 2008a; Kim *et al.*, 2010; Pereira *et al.*, 2012).

A trial of nutrient bioextraction technologies in Long Island Sound and the Bronx River estuary cultivating native strains of *Gracilaria tikvahiae* has been supported by the Long Island Sound

Futures Fund and the Connecticut Sea Grant College Program (Yarish *et al.*, 2012a,b; Kim *et al.* 2012). To grow seaweed in a nutrient bioextraction (or IMTA systems), it is necessary to have a source of young plants. In Asia, native species have been isolated from wild populations; strains have been selected for desirable traits and are maintained as “seed” cultures. Seaweed farmers often belong to a cooperative and obtain “seed” nets or lines from a seaweed culture facility (*i.e.* a seaweed nursery). This manual describes how to set up a seaweed culture laboratory, defines the basic resource needs of these plants, and provides a culture system roadmap for the production of young “seed” plants of the native red alga, *Gracilaria tikvahiae*.

General Seaweed Culture System Components

System

In order to cultivate seaweed, a simple but well-equipped laboratory is essential. The three most important components of a culture system are seawater media (seawater and nutrients), temperature and light. This chapter offers a broad overview for laboratory system components that will be applicable to all types of seaweed culture. For more information on algal culturing techniques, the reader is urged to consult Andersen (2005).

Seawater

Successful seaweed culture requires a reliable supply of clean seawater. Natural seawater is preferred over artificial seawater, as natural seawater contains all of the essential mineral components (over 50 known elements) in the natural ratios, which may or may not be available in artificial seawater. Seawater salinities should be between 28-34 parts per thousand (ppt), and it is critical that it be clean and free of any organic or inorganic contaminants. To avoid terrestrial runoff or salinity variation, natural seawater collection should occur during periods of dry weather, preferably at high tide, and at depths of approximately 3 meters (10 feet) below the surface. Seawater can be collected by boat or from a shore facility with a well-placed intake pump. Water storage and transfer can be done in polyethylene tanks or 20-liter (5 gallon) carboys. All new containers need to be leached before use by filling with clean water (ideally distilled water, but clean seawater can be used) and allowed to sit for several days before rinsing for use. All containers and tanks should be well sealed to prevent contamination, and kept in a cool dark place to prevent growth of unwanted algae.

Seawater should initially be filtered through a coarse filter during collection from the sea, using a sand filter or a canister filter, then further fine-filtered from there. Initial rough mechanical filtration can be accomplished with sand filters (pool filters) or polyester bag filters with 20-35 micron pore sizes. Further filtering can be accomplished with common household water filters, down to 0.2 microns, by pumping water through a series of canister filters of decreasing filter size (10-, 5-, 1- μm). A 0.45-micron filter will separate out organics from dissolved inorganics, and filtering water down to 0.2 microns should eliminate almost all possible biological contaminants in the water, though organisms can get through if there are tears or holes in the filters. For this reason, further sterilization of the water and periodic inspection of filters may be necessary to eliminate any contaminants that could threaten the culture (Fig 2).

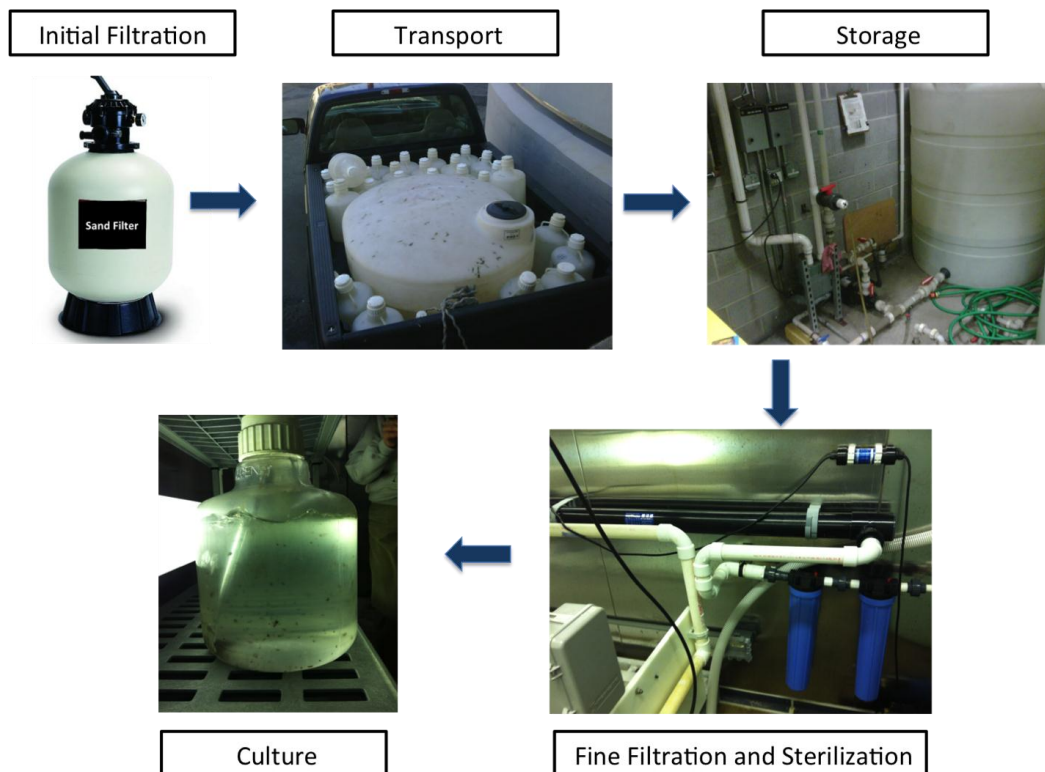


Figure 2 . Seawater system for culture. Initial filter, transport, storage, second filter, sterilization (UV, fine filter, or autoclave) culture.

Artificial seawater (AS) can be used, but caution is advised, for artificial mixes tend to have limited success compared to natural seawater. If it is necessary to use AS, a certain amount of experimentation may be required to determine the best source of mixed salts. They tend to be inconsistent, with great variability both between brands and within batches of the same brand. Artificial mixes may not have the same ratios of essential microelements present in natural seawater, which is why natural seawater is recommended for all culture work.

Sterilization

Once filtered, seawater should be sterilized, especially during culture initiation. While it is not necessary to have axenic (“pure”) cultures to grow seaweed, the microscopic and juvenile phases are sensitive to contamination. There are a host of microorganisms that can feed on, compete with, or overgrow the macroalgal cultures, including other algae, fungi, bacteria, cyanobacteria and micro zooplankton (primarily protozoans). Protozoan grazers can especially be a problem for the microscopic stages of the kelp and other seaweeds, if present at high concentrations. The only way to completely ensure sterility of seawater is through heat treatment.

There are a few different methods available to sterilize seawater, and the best method will depend on the facilities available. The most effective method of sterilizing seawater is by autoclaving, which ensures sterility through a treatment that applies extreme heat and

pressure to seawater (Fig. 3). Autoclaves are heavy walled closed chambers that can be used to sterilize liquids, metals, glass, or autoclavable plastics. Seawater can be sterilized in polypropylene, polycarbonate, or Pyrex containers. Containers should only be filled $\frac{3}{4}$ of the way, with loosely applied caps, to allow for pressure changes within the container during the heating and cooling of the liquid. Aluminum foil covers can be applied around the loose caps during the sterilization process to minimize exposure to air currents. A liquid cycle (121°C; 1-2 PSI, 15-30 minutes depending upon the volume of liquid) is sufficient to kill all living organisms, including bacteria, viruses, and heat resistant spores. In order to ensure the entire volume of liquid reaches the required temperature for a sufficient amount of time, containers should be allowed to equilibrate to the temperature of the warmed-up autoclave (30min-1hour) before running the cycle, and the cycle time should be increased for larger volumes of liquid. After the cycle has run, the door of the autoclave should not be opened until the pressure inside is the same as the room to avoid boiling over of the liquid.



Figure 3. Autoclave for sterilization of seawater and glassware.

Autoclaving can result in the formation of precipitates, especially phosphates, which are a critical micronutrient for seaweed growth. This occurs because the high temperature and pressure applied drives CO₂ gas out of the seawater, resulting in an increase in pH. Precipitation increases with higher levels of nutrients, so to avoid this, seawater is sterilized first, then nutrient solutions are added after it has cooled. Seawater should be removed from the autoclave when it is safe to do so, and allowed to cool at room temperature before storing in a cool dark place. It is best to store all sterilized water at 5-10°C to minimize chances of recontamination. After the water has cooled, caps should be completely tightened to avoid any chance of contamination through air exposure. Autoclaved seawater should be allowed to sit for several days before using or aerated with regular air to restore CO₂ equilibrium and lower pH levels. If a traditional autoclave is not available, a large pressure cooker may be used to apply heat and pressure to sterilize smaller amounts of water in the same way.

Seawater can be sterilized by application of heat only, through the processes of pasteurization and tyndallization. Pasteurization is accomplished by heating seawater up to a boiling point, to 90-95°C for 30-60 minutes, and then cooling rapidly to less than 10°C. Tyndallization applies the process of pasteurization three consecutive times to the liquid, over the course of three days. This method takes longer to accomplish, but provides extra treatment to kill heat resistant spores that will not be effectively destroyed in the first pasteurization. This can be

accomplished with a stovetop or a microwave oven, making sure that the seawater is well covered to avoid contamination.

Another way to sterilize water by application of heat is through flash sterilization. This will kill most organisms in seawater, but may not kill heat resistant spores. This method heats water up to a high temperature (70-90°C) in a very short time by passing through a tube or plate heat exchanger. This may not kill all possible contaminants, but can be an effective way to treat larger amounts of seawater.

Filter sterilization of liquid uses very fine filtration to exclude all living organisms, effectively sterilizing water without the application of heat. This method is used for small volumes of seawater or heat sensitive liquids, such as nutrient media and vitamin solutions. Liquid is filtered through a 0.2-micron filter, which, in theory, should exclude all organisms, though imperfections or defects in the filter pore size can allow some through. For small volumes, a reusable or disposable filter assembly is set up on a side arm flask, and liquid is pulled through the filter using a vacuum pump. Fine filtering can also be a part of a multi canister filter system, where seawater is pumped through a series of decreasing filter sizes.

A filtering system coupled to a UV light system may ensure extra treatment of seawater. Enclosed high-energy UV bulbs can be installed as part of the water filtration process. Filtered seawater passing through a UV sterilizer will be exposed to UV radiation, destroying any remaining organisms that may have passed through the filter. UV radiation may not be 100% effective, but can be an additional treatment to incoming seawater or water that cannot be autoclaved or heat sterilized.

Seawater can be sterilized with a chlorine treatment, by adding liquid bleach (5% sodium hypochlorite) to sterilize, and sodium thiosulfate to neutralize after treatment. After water is filtered to remove organic matter, bleach is added at concentrations of 1-5 mL of bleach per liter of seawater. Water should be left to stand for several hours, avoiding exposure to direct sunlight. After treatment, the bleach needs to be sufficiently neutralized before using. To neutralize, a sodium thiosulfate solution ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) is added at concentrations of 1 mL per 4 mL bleach added. The sodium thiosulfate solution is made by dissolving 250 grams of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in 1 liter of water (Kawachi and Noel, 2005). Neutralization of seawater should be verified with a chlorine test kit.

Table 1. Seawater sterilization options.

Seawater Sterilization Options			
Method	Temperature	Time	Notes
Autoclave	121°C	15-30 min entire volume	Containers filled $\frac{3}{4}$ Loosely applied covers
Pasteurization & Tyndallization	Heat 90-95°C, Cool 10°C	Heat 30-60 min, Rapidly cool	Repeat 3 times over 3 days
Filter Sterilization	n/a	n/a	Filter size 0.2-microns For heat sensitive liquids
UV Sterilization	n/a	Seconds of contact time	Most effective in recirculating water systems
Flash Sterilization	70-90°C	Rapid heating of seawater passing through heat plates	For large amounts of seawater
Chlorine Treatment	n/a	30 min – 24 hrs	Neutralize with sodium thiosulfate solution (1mL per 4mL bleach)

Nutrient Media

While natural seawater contains many of the necessary trace elements needed for seaweed culture, the quality and amount of nutrients can be variable and insufficient for culture work. Both natural and artificial seawater need to be enriched with a concentrated nutrient solution after the water has been sufficiently sterilized. There are several different types of media, but all provide a mixture of essential macronutrients (nitrogen, phosphorus, calcium, potassium, sodium, chloride, etc.) and trace elements (iron, manganese, zinc, molybdenum, copper, cobalt, zinc, etc.), metal chelators (EDTA), vitamins (B₁₂, thiamine, biotin), and hydrogen-ion and metal-ion buffers (TRIS, EDTA). Reagent laboratory-grade chemicals should be used to reduce the likelihood of contamination or impurities. Macronutrient, trace metal, and vitamin solutions are usually made up separately then added together to produce the concentrated stock solution. All solutions should be filter sterilized and refrigerated. Vitamin solutions can be frozen or refrigerated, but care should be taken to maintain sterility (Harrison & Berges, 2005).

Nutrient solutions are made up in large quantities beforehand, filter-sterilized into clean stock bottles, and refrigerated. It is useful to pre-proportion the nutrient solution into smaller bottles that are ready to be added to the volume of the culture tank to make seawater preparation easier. Preparation of enrichment solution requires a clean, sterilized table space, clean, sterilized glass and plastic ware, and sterile techniques. Only non-reactive metal instruments or glass should be used, as some latex and rubber can be toxic if leached into the media. In order

to filter sterilize media, self-assembly glass filter units with disposable filter papers (0.2 micron or μm) can be used. These units are placed into the mouth of a side-arm flask, and the liquid is pulled through the filter paper by suction underneath, created by a vacuum pump (Fig. 4). Instructions for preparation of this media can be found in Appendix A.

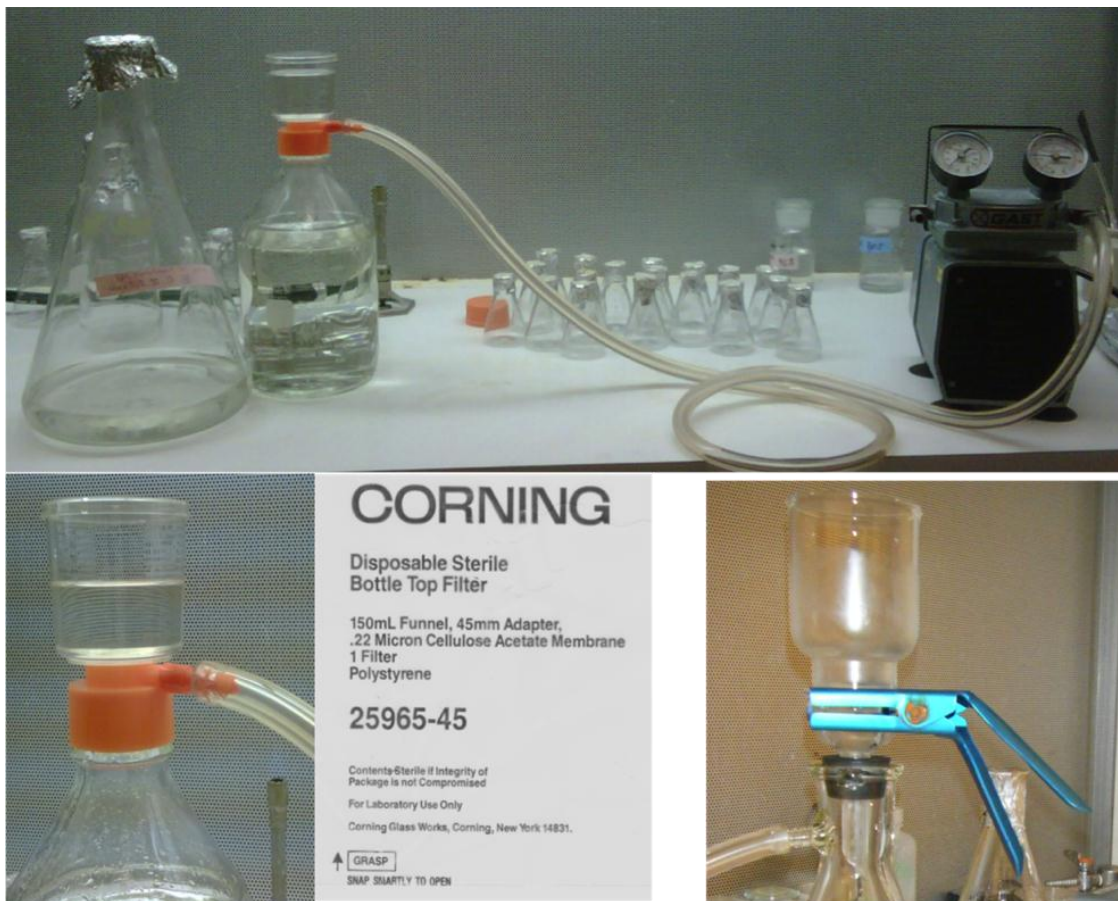


Figure 4. Vacuum pump assembly for filter sterilization (0.2 μm) of nutrient media (top). Filter assemblies can be disposable (left, bottom) or reusable (right, bottom).

Seawater media must be changed regularly during the culture period in order to provide sufficient nutrients to the growing plants. This is a simple operation that involves transferring plants from one culture vessel to a new one with fresh seawater media, taking care that the new culture water is at the same temperature as the old culture water to avoid stressing the seaweed. This should be done in a clean environment, using sterilized forceps and gloves, avoiding any chance of contamination through air currents or contact. Culture changes are usually done once per week or more in actively growing or dense cultures, and once every two to four weeks in cultures that are just being maintained and not actively growing.

Diatom Control

The most common and problematic contaminants in a seaweed culture are single celled microalgae called diatoms (Fig. 5). Diatoms are one of the most common types of phytoplankters, characterized by unique symmetric cell walls made of silica. Centric diatoms are radially symmetrical (round), while pennate diatoms are bilaterally symmetrical (shaped like a long thin football). Diatoms can exist as single cells, colonies, or filaments. They are able to rapidly proliferate through both asexual and sexual means, and tend to smother all available surfaces, including culture substrates and young blades. They are very likely to be introduced into cultures through natural seawater or on blades collected from the wild. Their small size and variable shapes allow them to pass through filtration systems and even UV sterilization. In order to control growth of diatoms in cultures of young seaweeds, a saturated solution of germanium dioxide is added to the culture media at 1-2 mL per liter of seawater. This compound inhibits silica deposition in diatoms (necessary for cell wall formation) and effectively eliminates diatoms in the culture (Lewin, 1966). Germanium dioxide should only be used when initiating cultures, and will not be a cost effective measure in large volumes of water.

Temperature

Water temperature control is a critical component in any seaweed nursery operation. Seaweeds have an optimal temperature range for growth, as well as a range of upper and lower survival temperatures (see Lüning, 1990 for a general introduction to environmental factors regulating seaweed growth and production). Cultures can either be placed inside a refrigerated space (cold rooms or incubators) or in individual tanks that may be cooled with an aquarium-type chiller (Fig. 6). If standard household refrigerators are available they can be converted into homemade incubators by installing fluorescent lights on the inside walls (make sure to install ballasts on the outside). Temperature alarm monitoring systems with automatic dialers can alert culturists in the event of a temperature failure (Omega Systems make a variety of these alarms monitoring devices).



Figure 5. Diatoms problematic in seaweed culture. Top: diatom embedded in kelp blade, second: pennate diatoms, third: pennate and centric diatoms, bottom: centric diatom. Scale bar = 10 microns.

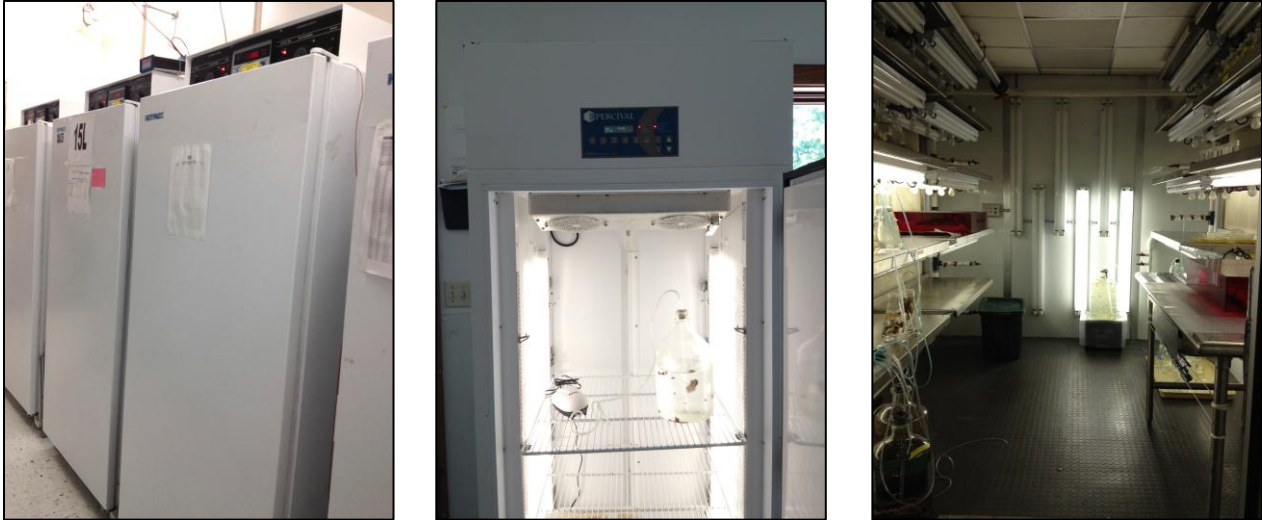


Figure 6. Environmental Culture Chambers, also known as incubators. These can be any size, and can be purchased commercially or can be homemade. Environmental chambers all have a means of temperature control, a light source with photoperiodic control, and aeration. Left: Hotpack brand Incubator, Middle: Percival Biological Incubator, Right: Room- sized environmental chamber.

Lighting

Seaweeds are photosynthetic organisms that incorporate inorganic materials into organic biomass using the energy of the sun. Seaweeds, like land plants, utilize photosynthetically active radiation (PAR), which is the portion of light available for photosynthesis. PAR comprises the same part of the light spectrum as visible light, and can be broken down into its colored components, from blue/violet (400nm, high energy) to red (700nm, low energy). The underwater light environment is highly variable and dynamic, because as light passes through water it is absorbed and scattered by water molecules and particles. The longer, lower energy red wavelengths are usually absorbed near the surface, while shorter, higher energy blue wavelengths tend to penetrate deeper into the water column. While all of the algal groups (green, red, and brown) contain chlorophyll *a* and carotenoids, they have different photosynthetic accessory pigments. Each type of pigment absorbs wavelengths in different parts of the spectrum, as shown below (Fig. 7), however, only certain wavelengths elicit a physiological response (often referred to as the action spectrum).

Light Absorption Spectra for Marine Algal Pigments

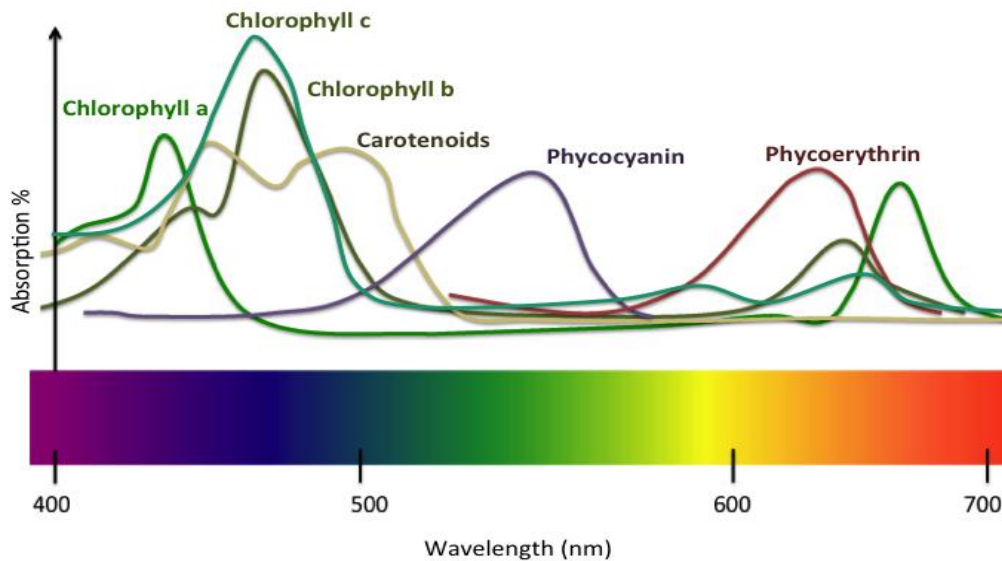


Figure 7. Light absorption spectra for photosynthetic pigments

For the laboratory, fluorescent light bulbs will provide sufficient light energy for the culture of plants. There are many different varieties of bulbs on the market, and the technology is ever evolving, so it is helpful to understand what type of light each bulb offers. Light quality and quantity are important when choosing a bulb. Higher output bulbs will provide higher light intensity or photon fluence levels. For this reason, the “high output” bulbs (or “HO”) are used in order to ensure enough light for the cultures. The quality of light available from each type of bulb is determined by the difference in the inside coating of the glass. There are three main types of bulbs, differentiated by their color temperature. The color temperature is a value in Kelvins printed on the bulb. Daylight bulbs have the highest color temperature, at 5000K or above, and are made to simulate natural sunlight. Cool white bulbs have more light in the blue, with a color temperature around 4000K. Warm white bulbs emit light in the red portion of the spectrum, with a lower color temperature of 3000K or less. Cool white bulbs are best, because most of the photosynthetic pigments in algae are able to absorb light in the blue portion of the spectrum (Fig. 8).



Figure 8. Fluorescent bulb color temperatures

To provide artificial lighting in the laboratory or indoor culture facility, cool white high output T12 fluorescent bulbs with electronic ballasts are best. However, more energy efficient bulbs

including T8 and T5 technologies are now replacing T12 bulbs. One note of caution, the spectral composition of all the bulbs are not equivalent, hence our reticence to use T5 bulbs vs the older T12 bulbs. The spectral compositions of all these bulbs are readily available by the manufacturers including General Electric, Phillips and Sylvania. Light can be provided from above or from the sides of clear containers, and several independently controlled bulbs can be used to increase or decrease light levels. Window screening can also be used to control light intensity by placing layers between the light source and the cultures, each layer of 1mm mesh screen resulting in a 40-50% reduction in light.

It is important to know the intensity of light available to plants, as light requirements change over time. Usually, new cultures require lower light levels, while developing plants require more light. Very low light levels (1-10% of normal culture conditions) can be used to maintain cultures over a long period of time. Quantum light meters are used to measure the amount of light available for algal growth. Light meters measure photosynthetic photon flux density, given in units of micromoles of photons per meter squared per second ($\mu\text{mol m}^{-2} \text{s}^{-1}$). Very good handheld quantum meters (*e.g.* Apogee Instruments, LI-COR) can be found on the Internet for order, with a wide variation in the cost, ranging from \$300 – \$2000.

Lights should be connected to an interval timer to control photoperiod. Photoperiod is the alternating period of light and dark, which is very important for normal development and growth of seaweeds. A neutral photoperiod is commonly used, which is 12 hours light followed by 12 hours of darkness (12:12, L:D). To establish the photoperiod, all outside light sources (such as windows or other light fixtures) should be eliminated. A long day photoperiod consists of long days and short nights, usually 14-16 hours of light followed by 8-10 hours of darkness. A short day photoperiod consists of only 6-8 hours of light followed by 16-18 hours of dark. Algae reproduction and growth is often triggered by changes in photoperiod and/or temperature, so manipulating photoperiod is a useful tool in a culture center (see Lüning, 1990, for further information on photoperiod).

Laboratory

Basic laboratory equipment for successful algal culture will include a microscope, a thermometer, a refractometer, a light meter, and some simple laboratory glassware.

A compound microscope is essential to determine successful spore release, spore density, and development of microscopic stages (Fig. 9). A high-powered dissecting scope is also very useful for monitoring larger plants throughout the culture period, or for isolating spores.

Microscopes should be kept covered with dust covers when not in use, and should be kept clean and dry. Microscope



Figure 9. Dissecting (left) and compound (right) microscope.

accessories include lens paper, Windex or alcohol, and glass slides and coverslips. A haemocytometer, specially etched for the counting of microscopic cells, is useful for spore solution density determination.

Some useful equipment to have in the lab will include a refractometer (Fig. 10), a light meter, a pH meter, a thermometer, and a magnetic stir plate. A refractometer is a simple handheld tool that measures salinity of seawater. A pH meter is required for culture media preparation, as well as for monitoring culture pH. pH meters need to be calibrated with buffer solutions before each use, rinsed before and after each use in deionized water, and stored in an electrode storage solution. A magnetic stir plate with stirrer bars is used for preparing nutrient media solutions (Fig. 11).



Figure 10. A Refractometer.

Glass and plastic ware for the lab will include volumetric flasks, graduated cylinders, beakers, flasks, and various culture dishes (Fig. 11). Borosilicate glass, Teflon, and polycarbonate are all common laboratory materials. Any material used in the lab should be non-reactive and non-toxic, and thoroughly cleaned before use. New glass or plastic ware needs to be pre-cleaned before use. This is done by washing, soaking in a dilute 10% hydrochloric acid solution, followed by a soaking in deionized water for at least 1-2 days. This step ensures the removal of any residue or chemical that could leach into the seawater or nutrient media. All lab ware should be carefully washed using laboratory detergent, rinsed in tap water, followed by a rinse in deionized water to remove any residual nutrients, or trace or heavy metals that can be present in tap water. All types of rubber, metal, or reactive materials should be avoided, and new tubing should be thoroughly rinsed before use. The size and type of glassware will depend on the needs of the grower. Glassware, brushes, and glassware detergents are available from laboratory supply companies. If buildup occurs on glassware with use, it can be soaked in a 10% hydrochloric acid bath for at least several hours, and then rinsed twice with deionized water. Acid baths should be prepared with proper protective equipment (goggles, gloves, and apron) and stored in a closed container to avoid evaporation. Any critical portion of the culture process should utilize only clean, rinsed, covered and sterile glassware to avoid any contamination. Washed, dried, and sterilized glassware should be stored in a clean cupboard, away from air currents.



Figure 11. Useful lab items (from top left to right): forceps, sterilized glassware, lab book, pH meter, stir plate, Bunsen burner, pipettes.

Glassware, tanks, and all laboratory equipment and working spaces should be clean and sterile to avoid contamination in cultures. Glassware and metal instruments can be sterilized by applying moist heat or dry heat. Moist heat is applied in an autoclave or pressure cooker, (1-2 bars of pressure at 121°C), making sure the steam penetrates the material, and caps (either aluminum foil or a plug) are applied loosely to containers. Dry heat can be used to sterilize glass or metal, by heating in an oven for 3-4 hours at 150°C. Aluminum foil is used to cap or cover equipment, ensuring that all openings are covered to maintain sterility upon removal from oven or autoclave.



Figure 12. Necessary laboratory equipment for successful cultivation: Left: sterile vacuum filter for making stock solutions, “Algal Culturing Techniques”, various sized Erlenmeyer flasks, beaker, refractometer, thermometer, graduated cylinder, Petri dishes, multiwall plates, air pump, and Pasteur pipettes. Right: Custom filter (10-100 micron mesh), squirt bottle with sterilized seawater, and glass stir rod with rubber policeman scraper useful for

A spray bottle of bleach solution or ethyl alcohol solution is very useful to have in the lab, for it can be used to sterilize and clean working areas and other equipment (Fig. 12). A clean working station will ideally be set up in a laminar flow cabinet (Fig. 13), but if this is not available, a clean room with minimal air movement is best. To set up a clean working station, the working surface should be cleaned with a microbial disinfectant (*e.g.* Amphyl[®] cleaner & disinfectant) or 70% ethyl alcohol solution before and after use. The ethyl alcohol solutions can be stored in small bottles or plastic containers. 95% solutions of the ethyl alcohol should be available to sterilize stainless steel tools (forceps, dissecting needles, blades). An open flame from an oil lamp or gas burner is also useful for sterilizing metal tools that are dipped in the ethyl alcohol solution. Parafilm[®], a plastic wrap used in laboratories, is very useful for covering and sealing containers, minimizing contamination and reducing evaporation of cultures.



Figure 13. Two types of clean hoods. Left, a biological safety cabinet, suitable for working with cultures or hazardous materials. Right, a laminar flow cabinet, suitable for working with cultures and non-hazardous materials.

A record of all cultures in the lab, nursery, or culture center should be kept in the lab with the source, history, and details of all of the cultures. This will allow the grower to track cultures over time. All cultures, chemicals, and seawater media should be well labeled, and appropriate federal and state laws should be followed in the handling, storage, and disposal of all chemicals and cultures.

Long-Term Maintenance

It is a good idea to maintain stock cultures of all cultivars in the lab. Stock cultures should be kept at lowered temperatures and light levels to minimize growth and need for culture changes. For long-term maintenance, cold-temperate plants should be kept at 5-10°C, warm temperate plants at 15-20°C. Illumination is best kept at 1-10% of normal culture conditions, which is at or near compensation level (generally 2-7 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

Gracilaria

Gracilaria tikvahiae

Gracilaria tikvahiae is the only *Gracilaria* species native to New England (Fig. 14). The genus *Gracilaria*, in the Phylum Rhodophyta (a group of the red seaweeds), contains over 100 species found around the world, and many are wild harvested and cultivated for food, animal feed, and the phycocolloid called agar. They are warm water seaweeds, usually preferring temperatures in the 15-30°C range, making them a potential summer species for culture in New England. *Gracilaria* is an ideal candidate for aquaculture due to its warm-water growing season, ease of propagation, relatively high growth rates, high tolerance to a range of environmental conditions, and its existing and potential commercial value.



Figure 14. *Gracilaria tikvahiae* in culture

Biology

Gracilaria tikvahiae has a variable morphology, which depends on the strain and growing conditions. It is a bushy, branching seaweed, comprised of rounded branches which are irregularly or dichotomously branched from rounded, compressed, or flattened axes (Fig. 15). Blades are usually red, but can be brownish, green, or almost black depending on light and nutrient conditions. Blades arise from a flattened disc that is formed from a spore. Growth occurs by an apical meristem, located at the tip of each branch. Blades can reproduce through spores or by vegetative propagation, and can be found as either attached or free floating in coastal areas.



Figure 15. *Gracilaria tikvahiae*

Gracilaria is a species that has a wide range of tolerance for changing environmental conditions. It is common in estuaries or bays, often found in intertidal or shallow subtidal areas, less than 1 meter deep, either attached to rocks or free floating. It is often found in embayments, which are environments with reduced water flow and may be rich in ammonia and nitrate. They are a euryhaline species, which means they can tolerate a wide range of salinities, from about 10-40 ppt, though they grow best in the 25-33ppt range. They can survive temperature ranges from 0-35°C but have an optimal range of 20-28°C.

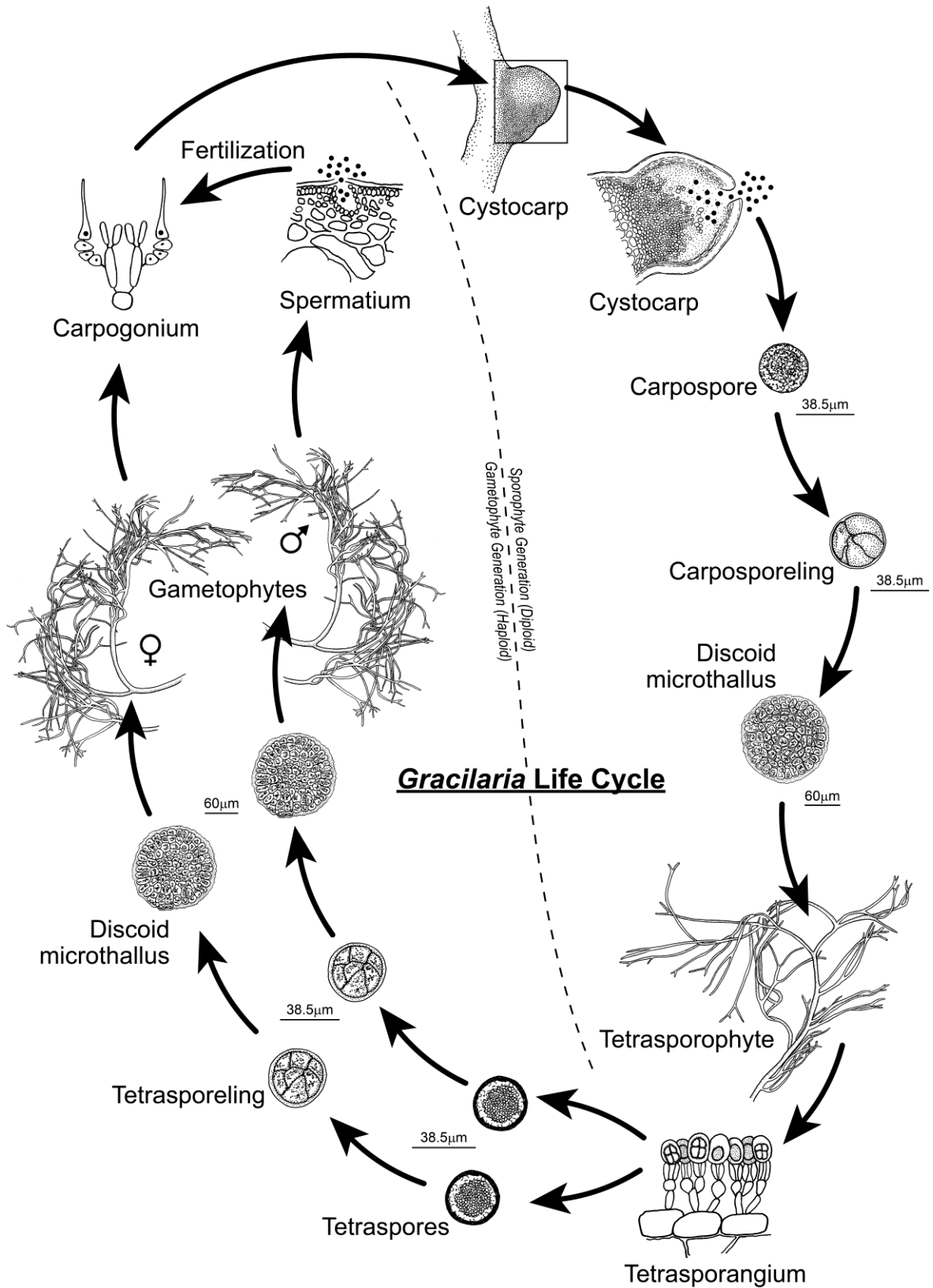


Illustration by Virge Kask, 2012

Figure 16. The *Gracilaria* life cycle.

Gracilaria has a three-stage life history that is similar to many other red seaweeds, often called a *Polysiphonia*-type life history (Fig. 16). It has an isomorphic (“same shape”) alternation of generations. This indicates that two of its three life stages, the tetrasporophyte and the gametophyte stage, are morphologically identical. The life cycle has three different stages; a diploid tetrasporophyte stage, a haploid gametophyte phase, and a diploid carposporophyte phase that occurs on the female blades. These stages are distinguishable only by microscopic examination of the reproductive structures or presence of the third stage, which appear as bumps on the branches of the female blade. The mature diploid tetrasporophyte produces four haploid tetraspores within each tetrasporangium by undergoing meiosis. Tetrasporangia occur in the cortex (the outer edge of cells) of the thallus, and can be found anywhere on the blade. The tetraspores appear as red cross-shaped (cruciate) spots, and can be observed with a microscope or even a hand lens. When tetraspores are released from the blade, they drift passively in the water column until they settle and adhere to a substratum. The spore will begin to internally divide, then enlarge and develop a multicellular disc. The center of this disc will then develop a raised dome and the blade will develop from this initial growth. Each disc may produce many upright thalli, each of which may separate from the disc and continue to grow as a free-floating plant.

The plants that develop from tetraspores are either male or female haploid gametophytes, indistinguishable until maturity. Mature male gametophytes produce white spotted areas with spermatangia on their thallus, observable under a microscope. Female gametophytes become apparent when their eggs (carpogonia), produced within the cortex of the plant, are fertilized by spermatia (non-motile sperm), and new tissue is built up around the zygote. The new diploid, globular structure is the third phase of the life cycle, the carposporophyte, occurring on the haploid female thallus. The small bump is a cystocarp, and inside the cystocarp, the original zygote undergoes many cell divisions (mitosis), eventually producing many diploid spores, called carpospores. These non-motile, spherical spores are released into the water column and carried to a suitable substratum by water currents. Once settled on a substratum, the spores will adhere, divide and form a multicellular disc. These multicellular discs will initially produce a protuberance that develops into an upright thalli, which will develop into a tetrasporophytes, thus completing the life cycle. Each disc may also produce many upright thalli, each of which may separate from their disc and continue to grow as a free-floating plant.

Besides the relatively complicated three-phase sexual reproductive life cycle, *Gracilaria* is also able to reproduce asexually, through vegetative propagation. A single individual has the capacity to become hundreds or thousands of individuals, through continual fragmentation. Each fragment produced will grow and develop into an individual, and these blades can be further fragmented into several individuals, and so on. This is possible because the growth occurs in the apical meristem, which is growth from the tips of the branches. Each tip, then, has the capacity to grow and branch into its own blade. This allows for a much simpler means

of propagation, and also creates consistency in a culture environment. Vegetative propagation is the most common means of culture, as it is quicker, easier, and more efficient than starting from spores, and it allows for consistency, as all blades in a culture can be genetically identical, all having the same parent (sometimes referred to as a clone). This is very important if the blades are being grown for a specific characteristic, such as agar consistency, specific morphology, or favorable growth rates and biomass yields. However, the vegetative propagation of a blade does not have an attachment stage, so the culture method needs to be adapted to the local needs and situation of the grower.

Cultivation

There are several different methods used for the cultivation of *Gracilaria*, and the ideal system for any one grower may be a combination of two or several of these following options:

Culture Initiation

- Tip Isolation
- Spore Isolation

Biomass Production

Asexual (Vegetative) Propagation:

- Tank culture
- Suspended Rope Culture
- Bottom Culture

Sexual Propagation:

- Spore seeded substratum (the use of carpospores or tetraspores)

Culture Initiation

To initiate a culture, it is necessary to establish a unialgal culture (containing no other alga) by either spore or tip isolation. Clean, healthy, actively growing, and/or reproductive 'parent' fronds exhibiting desirable characteristics should be selected. Fronds (sometimes referred to as thallus) may be chosen from wild populations or from existing cultures.

When making a wild collection, fronds should be held and transported in an environment similar to (or cooler than) the one it was collected in to minimize stress. Clean plastic bags, plastic containers, or buckets can be used for collection and transport. *Gracilaria* should be transported in moist paper towels or gauze to avoid drying and exposure. Regardless of the culture initiation method used, the critical step in culturing *Gracilaria* (or any seaweed) is the cleaning process. Any wild collected frond will be carrying a host of microscopic organisms

(diatoms, protozoans, fungi, other micro-, macro-organisms, or cyanobacteria) that can potentially contaminate, inhibit or destroy cultures. An initial rinse of the seaweed in the field can remove any visible fouling organisms, followed by a more thorough cleansing process in the lab. Whenever possible, it is recommended to make voucher herbarium specimens for more rigorous genetic study.

A successful culture is established in the lab with a healthy, actively growing, unialgal isolate free of any contamination. One successful culture can provide all of the ‘seedstock’ necessary to “seed” an entire farm. It is important to maintain a ‘seedstock’ culture in the lab for back-up and preservation purposes. To increase biomass from one isolate, fronds are fragmented and given sufficient light, space, and nutrients to multiply in number and size, and the process is repeated until enough biomass is reached to either “seed” a larger farm system or for direct harvest.

Tip Isolation

The area of new and active growth is located at the tips of every branch on a *Gracilaria* frond, the apical tissue. To obtain new ‘starter plants’ to initiate a culture, tips are cut from the parent frond, cleaned thoroughly, and placed in favorable growing conditions. Each tip will then grow, elongate, and branch into a new frond. To prepare a tip for isolation, a clean working area should be set up in a clean room with a draft-free, clean working space and a flame for sterilization of metal instruments to avoid any contamination of cultures.

Collected fronds from the field should be processed in a separate room from your culture isolation working space for the initial rinsing. The fronds are initially placed in the first container, and then fronds are individually selected and rinsed in a series of vessels by grasping with large forceps, submerging and shaking vigorously underwater several times. This can be repeated several times in a series of vessels where the last should contain the fronds ready for tip isolation. If there are still epiphytes present on the tissue, gentle scrubbing with cotton balls, cotton-tipped swabs, or paper towels can remove any clinging organisms. *Gracilaria* is a euryhaline algae (able to tolerate a wide range of salinities), so a quick rinse (30-60 seconds) in clean, deionized freshwater can be used as a final cleansing step. A small section of the frond can then be removed for tip isolation, and placed in a small dish with sterilized seawater and moved to the clean working space (Fig. 17).



Figure 17. Cleaning wild-collected *Gracilaria* (from left to right):

1) Initial rinse in clean seawater; 2) Selection of clean, healthy fronds and second rinse; 3) Scrubbing and selection of tips for isolation

To isolate tips, a clean, sterilized work surface is essential. The work area should be set up with sterilized jeweler's forceps (fine tipped tweezers), a sharp scalpel or razor blade, ethanol, and a flame source. A small section of the parent frond is placed in a Petri dish with sterile seawater, and healthy tips are excised with a scalpel or razor blade. Once all tips from a section are cut, the excess frond is removed and the tips are further cleansed. It is useful to cut the tip a little longer than what is needed, about 1mm, in order to grasp the cut end of the tip with jeweler's forceps (Fig. 18).

Once a number of healthy tips have been excised, each tip is individually cleaned in sterile seawater. A series of Petri dishes can be set up in the working area to clean individual tips. A tip is placed in sterile seawater, grasped at the cut end with fine tipped forceps, and wiped down with a sterile cotton-tipped swap. This is followed by an agar drag through a prepared agar plate (see index for instructions), which will pull off any additional microscopic contaminants. A scrubbed tip is grasped, again, at the cut end, and dragged through the agar gel at least three times. Each drag should go through an unused portion of the agar gel. When the agar gel is used up, plates should be properly disposed of. The tip should now be free of contaminants, and can be placed in sterilized seawater prepared with Von Stosch's Enrichment media (VSE) and germanium dioxide (GeO_2). Multiple tips should be isolated from each frond in order to increase chances of obtaining a clean culture, because even all of these careful steps will not guarantee a unialgal culture. To maximize success, always check your culture under a microscope.



A. Excising Healthy Tips



B. Tip collection



C. Agar Drag for cleansing tips



D. Initiation of culture from tips



E. Scaling up cultures



F. Scaling up cultures

Figure 18. Isolating and cleaning tips for establishing a unialgal culture.

Individual tips can be isolated and cultured separately in small Petri dishes or flasks. They should be placed in VSE seawater with GeO_2 under low light ($10\text{-}20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), with a 12:12, L:D photoperiod, at 20°C ($=68.8^\circ\text{F}$). Lower light may discourage the growth of any remaining epiphytic contaminants. Tips will begin to elongate in about a week or two. Once tips have begun to grow and appear clean, light aeration can be applied to cultures to increase growth rates. Cultures should be changed once every two weeks initially, then once per week as growth rates increase. Once tips begin to elongate and branch into larger fronds, they should be transferred to larger and larger containers to encourage growth (Fig. 18).

Once a clean culture is established, it can be expanded through fragmentation, by breaking up one frond to start many new fronds with the fragments. The growing environment is optimized to increase growth rates by gradually increasing light levels (up to $250 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), increasing growing area (larger containers/tanks), and increasing frequency of media changes (to increase availability of nutrients). Clean glass culture bottles, jars, flasks or carboys of varying sizes are excellent for expanding or maintaining your cultures. Once a sufficient biomass is reached, the fronds can be transferred to larger indoor or outdoor tanks to expand further. To preserve or maintain original culture strains over long periods of time in the laboratory, growing environments are minimized, reducing light, reducing temperature, space, and frequency of media changes.

Spore Isolation

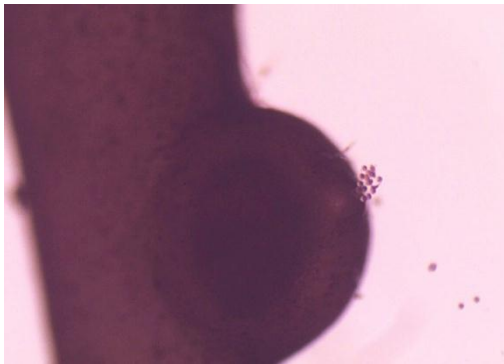


Figure 19. Carpospore release from a cystocarp formed on the surface of a female thallus.

Clean cultures can also be initiated from either carpospores or tetraspores. Both types of spores can be released and isolated in the same way, though each will give rise to a different phase in the life cycle. Carpospores are obtained from mature cystocarps, which are apparent as bumps on the female thallus (Fig. 19). These are easily identifiable, being obvious without the aid of a microscope. A microscope, however, is needed to observe the presence of the

tetrasporangium on the thallus of the tetrasporophyte. These appear as small reddish spots scattered throughout the cortex of the thallus.

To release spores, it is important to clean the parent frond well by shaking, scrubbing, and rinsing in sterilized seawater. Reproductive branches can be removed and wiped clean with a cotton-tipped swab and followed by an agar drag. A gentle desiccation period can be utilized to stimulate release of spores by wrapping the branch in damp paper towels, placing in a Ziploc[®] bag, and storing for a few hours or overnight in darkness or dim light. If fronds are mature, the desiccation period can be skipped and placed in seawater for release. To release spores, short sections of the fronds can be placed in sterilized seawater in a Petri dish over glass slides and

kept under low light at 20°C. Release may take place at once, or can occur over several days. Spore release can be checked under a dissecting microscope.

Once there are spores released in the water, a small sample can be removed from the Petri dish and placed in a fresh dish with new media. Individual spores can then be selected with a very fine-tipped Pasteur pipette under a microscope. Selected spores are placed on cut glass slides (25mm x 25mm) or on coverslips in small Petri dishes with sterilized VSE seawater. Dishes can be kept undisturbed at 20°C under 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light, with a 12:12, L: D photoperiod. Spores will settle within 12-24 hours after release, adhere to the glass slide, and begin to divide. After the initial division, the diameter will begin to increase as a multi-cellular disc is formed, a few days after settlement. The center of the disc will then undergo further cell division to create a raised dome in the center, but this is a slow process, taking up to 2 months to form a 2-3 cm cylindrical axis. This is the beginning of the new frond (Fig. 20).

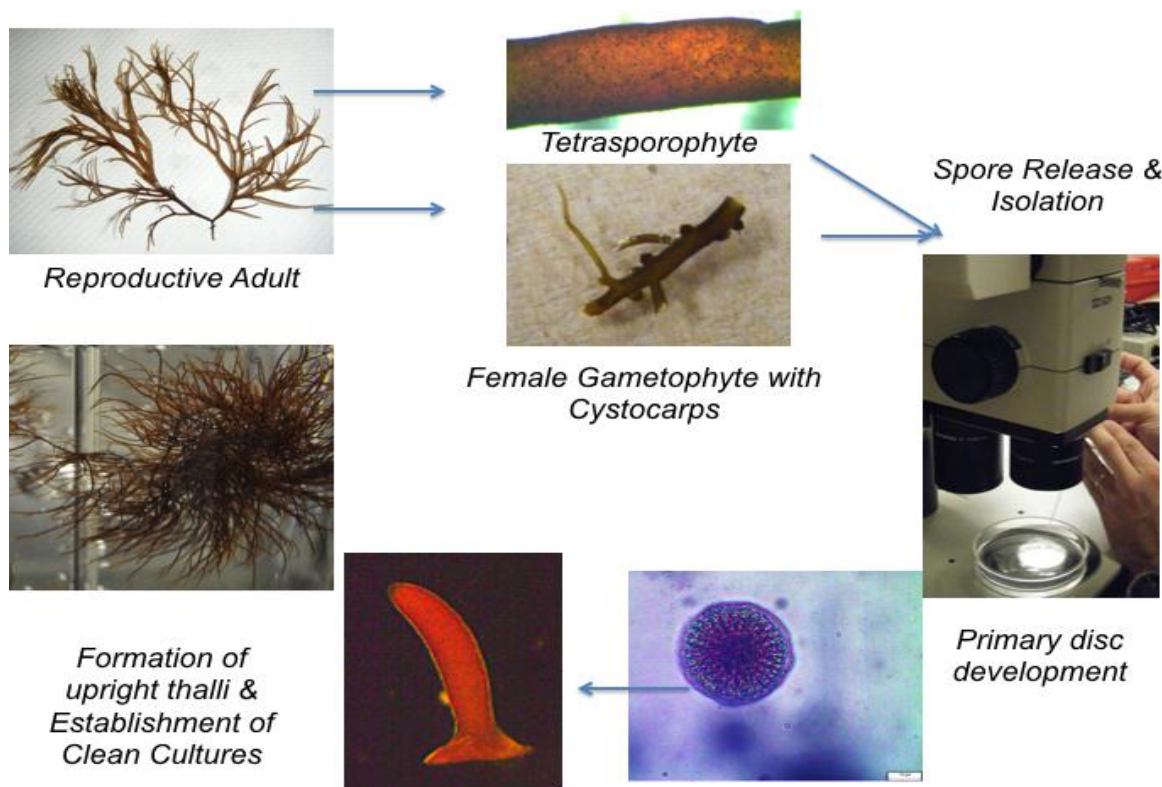


Figure 20. Establishment of clean *Gracilaria* laboratory cultures from spores

Spore initiation will result in an attached frond, and this can easily be transferred to new dishes by moving the glass slide, or the disc can be carefully scraped off the slide for an unattached frond. Once the first shoot begins to grow, light can slowly be increased, aeration can be added and sufficient water changes and larger containers can increase growth rates (Fig. 21). While spore isolation reduces the opportunity for contamination, the period of development is much longer than tip isolation. It can take 2-3 months to reach the size of an isolated tip. Spore

isolation allows the grower to start new cultures from a known point, however, for more control over their cultures. If a culture is started from a carpospore, the grower can be certain that the resulting frond is a tetrasporophyte, and if started from a tetraspore, it is certain that the resulting frond is a haploid gametophyte, though it is impossible to determine the sex until the frond is mature.



Figure 21. *Gracilaria* lab culture. Establishing new fronds and building biomass.

Biomass Production

Asexual Propagation

The most common method of *Gracilaria* cultivation is through vegetative propagation. This is a simple process that allows the grower to start many new fronds from the tips of any single frond. This is a form of clonal propagation, where all of the new fronds started from the initial “parent” will be genetically identical, which is useful for consistency in production. This is probably the easiest form of propagation, but it results in unattached, free floating fronds, which lends itself well to tank cultivation, but presents some challenges for any type of attached culture.

Culture Systems

Tank Culture



Figure 22. Building biomass for *Gracilaria* culture: Laboratory scale, small tanks, medium tanks, large outdoor seawater tanks.

Gracilaria does very well in a tumbled tank culture system (Fig. 22). This is due to its ability for unattached vegetative growth over long periods of time, ease of propagation, and high growth rates under ideal conditions. In this type of system, fronds are stocked in a tank of appropriate size and are given light, nutrients, and aeration. This type of system is intensive, requiring an input of energy (light, nutrients, and water movement) for culture. While tank culture may require more input energy, it allows for full control of the growing parameters as well as contamination control. It is the best method for developing 'seedstock' for further out-planting, and will result in the highest quality fronds for edible or cosmeceutical/nutraceutical/pharmaceutical markets.

Aeration is a critical component of the tank culture, and is responsible for delivering several critical elements to the system. Aeration should be well distributed throughout the tank to deliver sufficient water movement to constantly 'tumble' the fronds around the tank. This allows for a higher stocking density, as the constant movement reduces shading limitations, increases light exposure, and increases photosynthetic efficiency throughout the culture.

Aeration also increases availability of carbon dioxide and stabilizes the pH, which can rise to very high levels in an actively photosynthesizing culture. Water movement created by aeration simulates natural water currents, which are important for delivering nutrients to the surface of the fronds. Seaweeds will take in nutrients from the surrounding water, and if there is no water current to replenish the water around the thallus, a “boundary layer” of nutrient-depleted water will be established, and growth will be inhibited. Water current is very important for sufficient nutrient delivery to all fronds in any culture, especially an intensive culture with a high stocking density. Current can also be important for reducing contamination on the fronds, as it may make it more difficult for other organisms to settle on the surfaces of the fronds if the water around the fronds is turbulent.

Any type of air blower can provide aeration, though the air should be filtered before entering the cultures (Fig, 23). The distribution of air can be established by running rigid tubing or PVC pipes throughout the tanks to create full circulation. Air should be moving up from the bottom of the tanks on the outside and the center of the tank to ensure there are no ‘dead spots’ where fronds could accumulate.

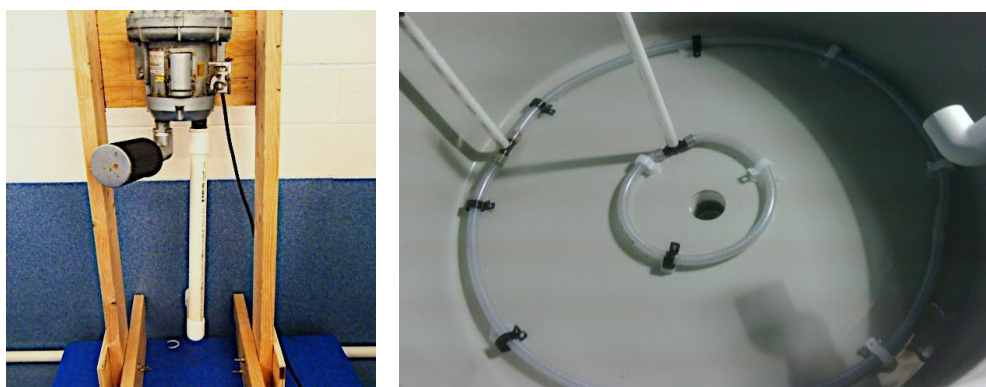


Figure 23. Aeration system for tank culture. A blower with air filter and aeration tube arrangement for a round tank.

The addition of sufficient nutrients to a culture is essential to obtain maximum growth rates and biomass yields. In a tank culture, this can represent a system expense, though the coupling of seaweed tanks with other ‘waste’ streams for alternative nutrient supplies is a possibility, as *Gracilaria* has a high bioremediation potential. Some alternative nutrients may include waste from other cultured animals (fish or shellfish), sewage, or fermentation residue from anaerobic digesters. In the absence of an alternative fertilizer, conventional land-plant fertilizer may be added. Fertilizer should be added in small daily increments to avoid ammonia toxicity. *Gracilaria* is able to take up nutrients in excess of their immediate needs and store these ‘reserves’ in their tissue for use in nutrient depleted situations. This storage capacity is useful to the farmer, for fronds can be ‘fertilized’ by soaking in high-nutrient media for 6-12 hours, then transferred to another site that may be low in nutrients. The seawater media in a culture should be changed on a regular basis to remove possible contaminants and to provide fresh nutrients. Larger cultures can be monitored for nutrients levels and half of the water volume changed on a regular basis to conserve inputs.

Light should be provided to the culture system in such a way to maximize growth while minimizing cost. Initially, lighting inside the laboratory can be provided with fluorescent lights or greenhouse growing lights, and light should be placed on a photoperiodic timer. As the culture system is expanded, natural lighting should be utilized, though fronds should be protected from extreme exposure by covering with neutral density screening. A light meter is an essential tool for determining and adjusting light levels as appropriate. Light (given non-limiting levels of nutrients) is an important parameter for controlling pigment levels in *Gracilaria*, and a controlled environment allows the grower to adjust light levels for desired pigment levels or the production of photo-protective pigments. This may or may not be important, depending on what the final product is being sold for. Light, aeration, and nutrients all work together to produce the final product. It is important to ensure that all fronds are exposed to enough light through constant turn over and tumbling, and nutrients need to be provided at non-limiting levels to allow for optimal growth rates, given sufficient light.

The culture is easily expanded by breaking apart, or fragmenting, the fronds, which will continue to branch and grow into new fronds (Fig. 24). The culture should be kept at a density that will maximize growth with efficient use of the resource inputs (e.g. 2-4 g L⁻¹). As the culture expands, the density can be reduced by removing fronds or by moving to a larger container or tank. This is an efficient way to grow up a large amount of initial biomass for further cultivation in a field setting or in larger tanks. For more in-depth discussion of lab and tank rearing technologies see Craigie and Shacklock (1985), Craigie (1990) and Craigie *et al.* (1999).

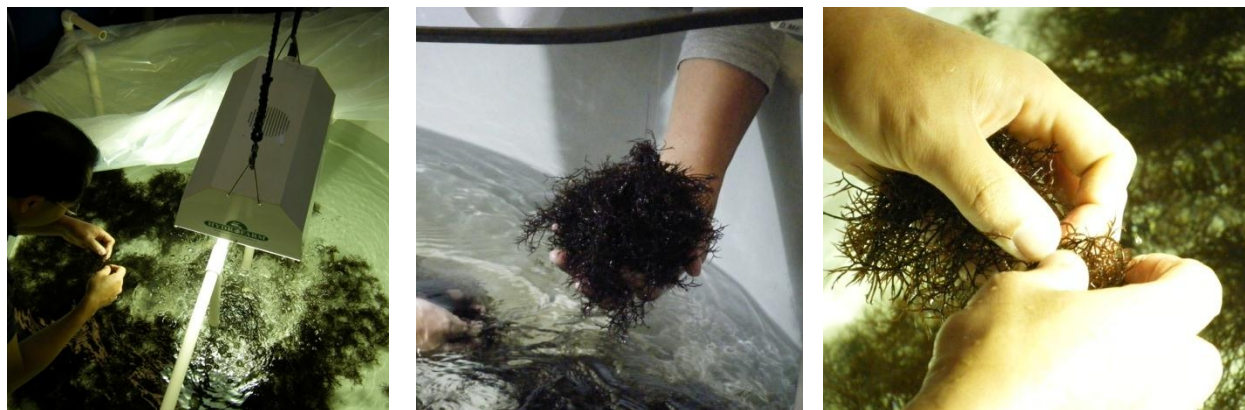


Figure 24. Building biomass in a tank culture through fragmentation of fronds.

Suspended Rope Culture

In order to reduce input costs of culturing seaweeds, field culture is an option, wherein seaweed fronds are placed out in the coastal environment to take advantage of the available natural resources. This is an extensive farming practice, where inputs and labor are relatively low, compared to the highly controlled intensive laboratory and tank culture. Suspended rope culture is a relatively simple fixed grow-out system, comprised of a floating longline held in place by anchors and buoys. *Gracilaria* plants are attached to the line by entwining or tying, and are further grown out on the lines (Figs. 25, 26). *Gracilaria* can be outplanted on longlines when temperature rises above 15 °C (60 °F). As biomass increases, fronds can be harvested by ‘trimming’ the outer portion of fronds and leaving fronds to re-grow to cut again every two to four weeks, or by a total harvest, by taking in the whole frond. Culture sites will vary considerably in their conditions, and local prevailing light, temperature, nutrient and salinity conditions should be determined for optimal growth (Fig. 27).



Figure 25. *Gracilaria* bundle inserted in line for field culture.

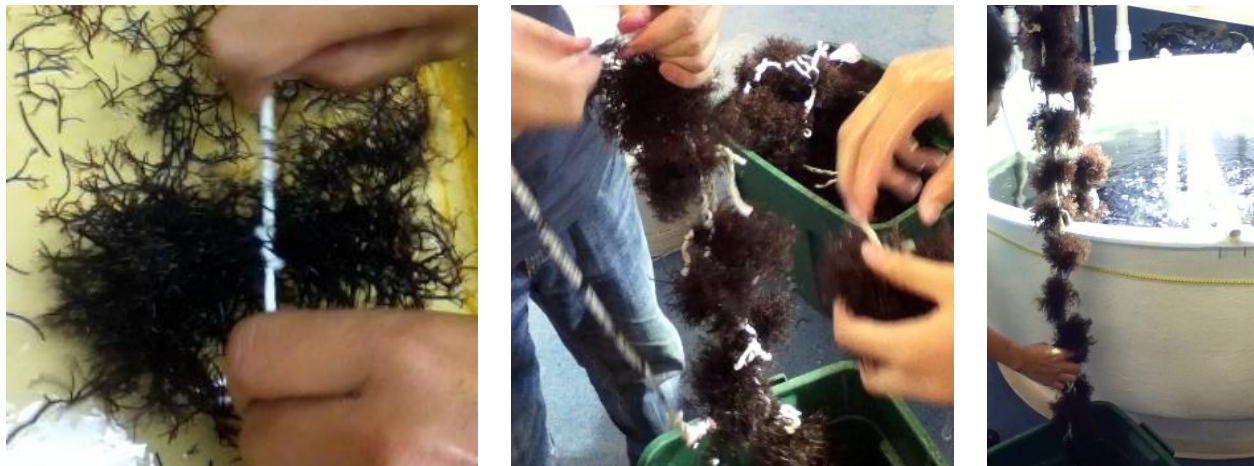


Figure 26. Inserting *Gracilaria* bundles onto a longline for out-planting.

Problems associated with field culture of *Gracilaria* include grazing, fouling, and environmental stress. Grazing by herbivorous fish or other predators is not as much of a problem in the Northeast as in the warmer climates, but smaller grazing animals are a possibility. Fouling is the biggest challenge in an open water grow-out situation. Many different types of fouling organisms may settle on the lines, including epibionts, such as tunicates, hydroids, bryozoans, mussels, worms, and amphipods, and epiphytes, such as other green, red, and brown seaweeds

or microalgae. There are several methods available for minimizing fouling. These include controlling depth, stocking density, and out-planting or harvest time. Depth can be adjusted, either up or down, to minimize settlement or survival of particular organisms, which tend to be more abundant at a particular depth. Increasing stocking density and maximizing growth rates will allow the fronds to outcompete or exclude potential fouling organisms. All organisms in the Northeast, including fouling organisms, have a seasonally controlled life cycles. Once the life cycle is understood, timing the outplanting of the seaweed lines and harvest of the crop can be a very important method for avoiding fouling settlement windows.

Extreme weather events can pose a hazard to the farm, either by causing damage to the infrastructure or by stressing the fronds. Farms should be sited in protected areas, and should be strong enough to withstand storms or high wind events. Flexibility in the design of a farm is important to minimize losses. An example of farm risk management would be the ability to bring lines in for storage in holding tanks during dangerous storms, or being able to adjust depth so that lines could be lowered to avoid extreme wave action or runoff events. *Gracilaria* is a stress tolerant species, but extreme changes in light, salinity or temperature can stress a culture unit and reduce growth and production.



Figure 27. *Gracilaria* open water farm (left). Deployment and cultivation of *Gracilaria* on longlines in the open water farm (middle). The harvesting of *Gracilaria* bundles.

Bottom Culture

Bottom culture is a popular culture technique in warm temperate climates, but may be difficult in the Northeast due to leasing area limitations. Bottom culture involves either growing fronds that are ‘seeded’ onto rocks and spread on the bottom in a shallow area, or by attaching fronds to lines that are strung on stakes and suspended just above the bottom. The technique of suspending lines from stakes is very similar to the suspended line culture, but is a little less flexible with depth adjustment. Access, however, may be easier in shallow sites.

Sexual Propagation:

Spore seeded substratum (carpospore or tetraspore)

Spore seeding is an alternative means of *Gracilaria* propagation. Instead of fragmenting a frond to produce new fronds, spores from a mature carposporophyte or tetrasporophyte are seeded onto a substratum, allowed to develop into juvenile fronds, and placed out into the growing area. The advantage of spore seeding is the convenience of attached fronds, while the disadvantage is the long incubation period to grow the settled spores into young fronds. The development of a juvenile frond 1-3cm in height can take two months in culture. Spore-

originated fronds can also exhibit a higher level of polymorphism, since all fronds are not genetically identical, as with the asexually propagated fronds. This will increase capacity for adaptation and survival in fluctuating or changing environments, but may not be ideal for a specific commercial purpose.

Spores can be released over the desired substrata (seed line or rocks) by laying prepared, mature spore-bearing thalli on a screen suspended in water. Spores are released over a 2-4 day period at 20°C, and once released, spores are allowed to settle undisturbed for 24-42 hours in dim light. If seeding both sides of a line wound on a frame, the frame is inverted and the same release and settlement period is done for the other side. The seeded substratum is then kept at 20°C under low light (20-40 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) for approximately two months for development of the juvenile fronds. Culture media (VSE) should be changed every two weeks to start, then more often as fronds start to develop. Gentle aeration can be applied for water movement. Once juvenile fronds are visible, they can be transferred to the culture site. A protected site should be chosen to avoid frond loss in high-energy environments.

Spore seeding of rocks may be a way to reseed or restock natural populations of *Gracilaria*. Once the fronds are established, the seeded rocks can be broadcast on the bottom of a protected shallow area, in order to help reestablish natural populations or supplement harvests.

Mixed Culture Methods

Several different culture methods may be employed in the culture of *Gracilaria*, and best practices will depend on the facilities available to the grower. Fronds grown in tanks may be used to stock long lines, and additional spore seeding of ropes may be possible to supplement the harvest and increase productivity. Other types of grow out systems are possible, including net tubes instead of lines, or floating cages stocked with unattached fronds. A 'spray culture' is another option, where recirculating water is sprayed continuously over fronds that do not have to be submerged in a tank of seawater. Fronds grown in areas or time periods of low levels of nutrients in the field can be 'fertilized' by placing in land-based tanks with high levels of nitrogen for 6-12 hours, then placed back into the field. Successful culture and production will depend on the grower's flexibility, inventiveness, and good management practices. For more in-depth discussion of cultivation techniques the reader is urged to consult Yarish and Pereira (2008).

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Appendix

Lighting

- Light Resources: Bulbs, Information, and Distributors
- GE Lighting: www.gelighting.com
- Philips: www.usa.lighting.philips.com
- Sylvania: www.sylvania.com

Handheld Quantum Light Meters

- Apogee Instruments www.apogeeinstruments.com
- LI-COR Environmental www.licor.com

Light measurements

- $\mu E = \mu\text{mol photons m}^{-2} \text{ s}^{-1} = (\text{lux})(0.013)$
- Light measurements conversions http://www.egc.com/useful_info_lighting.php

Supplies

Artificial Sea Water

- Instant Ocean (www.instantocean.com)
- Tropic Marin Sea Salt (www.tropic-marin.com)
- Ultramarine Synthetica www.waterlife.co.uk/seaquariums/ultramarine.htm
- Sigma-Aldrich Dry Sea Salt Mixture (www.sigmaaldrich.com)

Pre-mixed Enrichment Stocks

- National Center for Marine Alga and Microbiota NCMA (formerly CCMP) <https://ncma.bigelow.org/>
- Culture Collection of Algae and Protozoa (CCAP) www.ife.ac.uk/ccap
- Sigma-Aldrich f/2 media (www.sigmaaldrich.com)
- f/2-AlgaBoost, ES enrichment stocks (www.algaboost.com)

Aquarium Equipment & Supplies

Tanks, pumps, aeration, tubing, filters, lighting, etc

- Deep Blue Professional (www.deepblueprofessional.com)
- Aquatic Ecosystems Inc (www.aquaticeco.com)
- Lifeguard Aquatics (www.lifeguardaquatics.com/)
- Frigid Units, Inc (www.frigidunits.com)
- Emperor Aquatics, Inc. (www.emperoraquatics.com)
- Aqualogic (www.aqualogicinc.com)
- Polytank, Inc. (www.polytankco.com)
- Ironfish Aquaculture Directory (www.ironfish.org)

- Percival Scientific (www.percival-scientific.com)
- Omega Engineering Inc. (www.omega.com)
- Hydroponic and Greenhouse Suppliers (www.sunlightsupply.com, www.hydrofarm.com, etc)

Laboratory

Agar Media for Preparation of Agar Plates

- To prepare sterile agar plates, use new disposable plastic Petri dishes or sterilized glass Petri dishes.
- An agar solution is prepared by adding 1.5% agar powder to a 50:50 sterilized seawater/fresh water solution in a flask.
- The media should be microwaved or heated on a stir plate to both fully dissolve the agar powder and to sterilize the solution (about 5 minutes). Cover the opening of the flask with tinfoil upon removal.
- Prepare a clean working space free of any air currents to pour hot agar into plates. An open flame in the working space is used to sterilize the neck of the flask by passing it through the flame before pouring.
- While pouring the agar solution, open the Petri dishes as little as possible, holding the cover at an angle, and keep the lid over the dish. Cover the poured dish. Place agar plates on an undisturbed surface to cool and set. Agar medium will set into a stiff gel at room temperature.
- Stack cooled and hardened agar plates upside down in the refrigerator. Do not freeze. Plates are stacked upside down to prevent condensation from dripping down onto the agar surface.

Haemocytometer counts for cell density

A bright line haemocytometer is a specially etched glass slide made for taking blood cell counts, but is also useful for spore density calculations because it allows for an estimation of number of cells per milliliter of spore solution. The center of the haemocytometer slide contains two loading wells, each leading to a number of etched blocks on top of the slide which are apparent under the microscope. A haemocytometer comes with a special cover slip, and this should be placed over the center of the etched glass. There are two small wells on both sides



Haemocytometer and coverslips

of the glass slide, under the cover slip. These are loaded by placing a well-mixed drop of the sample solution in each well with a fine tipped pipette. The drop containing the spores will be pulled over the series of blocks, each with a different grid pattern. The middle block is divided up into 25 gridded squares, representing 10^{-4} ml. This is the block that should be counted. The haemocytometer should be viewed at 100x or 200x, and one square at a time should be counted. It may be easier to view the etched blocks under the microscope by turning down the light and closing the aperture of the microscope to increase contrast. The eyes should be methodically moved from the left to the right, and a consistent way of counting the cells on lines should be established. Cells on dividing lines are only counted in each square from the top (or bottom) and the left (or right). Consistency is very important for getting cell counts. A handheld clicker can be used if the density is very high, or the solution can be diluted by adding more seawater to count a more manageable sample.

Density Calculation:

Count the number of spores found in the 25 blocks that make up the middle block of the haemocytometer. This number represents the amount of cells in 10^{-4} ml, so to find the density of spores per ml, just add 4 zeros to your count.

Example:

Count = 40 spores

Just add 4 zeros to your count = 400,000 spores / mL

Now to find the total amount of spores available, multiply this by the total amount of spore solution that you have: Ex: $100\text{ml} * 400,000 \text{ spores} = 40,000,000 \text{ spores} / 100 \text{ mL}$

To determine the amount of spore solution to add to your spools for inoculation, determine the total amount of seawater being used for the inoculation:

Ex: 6 Liters = 6,000 mL

Spools should be inoculated at 2,000-5,000 spores/mL. To find the total amount of spores needed for your inoculation, multiply your desired density by the total amount of seawater being used.

Ex: 2,000 spores/mL * 6,000 mL = 12,000,000 spores total

Now you can divide the total amount of spores by the number of spores per mL in order to determine how many mL of spore solution you should add to inoculate your spools.

Ex: 12,000,000 spores / 400,000 spores ml⁻¹ = 30 mL spore solution

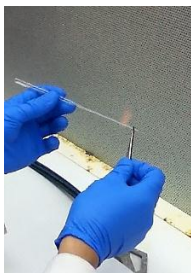
Germanium Dioxide

Diatoms are a common type of contamination in seaweed cultures, but can be eliminated with addition of a saturated solution of germanium dioxide to culture media. A saturated stock solution can be prepared by dissolving 250mg of GeO₂ per 1 Liter of deionized water. This stock solution is then added to culture media at a concentration of 2mL/L seawater. Stock solutions should be stored in a refrigerator and properly labeled. Brown algae are also sensitive to high concentrations of GeO₂, so this concentration should not be exceeded when culturing kelp. One to two weeks of treatment is usually sufficient to eliminate diatoms in a culture.

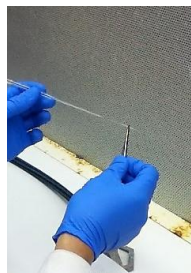
Micropipette Preparation

Micromanipulation by micropipette allows for the selection and isolation of microscopic spores under a microscope. Micropipettes can be prepared in the laboratory using disposable glass Pasteur pipettes.

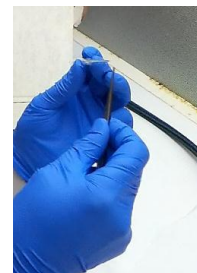
Holding the top of the pipette in one hand, and the small-bore end with a pair of steel forceps, hold the end of the pipette over an open flame to soften the glass until malleable. Take the pipette out of the flame and pull the ends apart, stretching the glass to form a very small bore. Snap the end off, and carefully place aside. Several of these micropipettes can be made up beforehand for isolation work under the microscope.



1) Hold glass pipette over flame until soft



2) Pull ends apart to stretch glass



3) Snap off end

Nutrient Media

VON STOSCH'S ENRICHED SEAWATER MEDIUM

von Stosch's Enrichment (as cited by Ott, 1966)

The seawater should be filtered (Whatman's #1) to remove large organic particles and sand. Then sterilize by autoclaving (time: 100 ml requires 10 minutes; 2 liters requires 40 minutes; 3 liters requires 50 minutes; and 5 liters requires 70 minutes). To each liter of seawater, then add the following:

Salts	1 liter of seawater
(1) Na NO ₃	42.50 mg
(2) Na ₂ HPO ₄ 12H ₂ O	10.75 mg
(3) FeSO ₄ 7H ₂ O	278.00 ug
(4) MnCl ₂ 4H ₂ O	19.80 ug
(5) Na ₂ EDTA 2H ₂ O	3.72 mg

Vitamins	
(6a) Thiamine-HCl	0.20 mg
(6b) Biotin	1.00 ug
(6c) B12	1.00 ug

It is convenient to prepare a stock solution of each salt in distilled water; of such concentration that 1 ml of the stock solution gives the required concentration of each ingredient. The three vitamins may be incorporated in the same stock solution, which should be refrigerated. The salts and vitamins after preparation into stock solutions should be filter sterilized.

- I. To make stock solutions use deionized distilled water and clean volumetric flasks.
- II. Filter each stock solution through separate 0.22 um Millipore filters. Each solution will have to be sterilized separately.
- III. Aseptically pour filtered volume of liquid into autoclaved stock bottles.

<u>1 liter stock solution</u>	<u>2 liter stock solution</u>
(1) 42.500 grams	85.000 grams
(2) 10.750	21.500
(3) 0.278	0.556
(4) 0.0198	0.039
(5) 3.720	7.440
(6a) 0.200	0.400
(6b) 0.001	0.002
(6c) 0.001	0.002

Modified Von Stosch Enrichment (VSE) for use with red algae

****To sterilized seawater, add 1 mL per 1 L of seawater of prepared solutions 1-6 after combining 3 and 4 together prior to addition****

<u>Solution Components</u>			
Ingredients			
<u>Solution 1: Nitrogen</u>	<u>Quantity (grams)</u>	Quantity (X2)	Quantity (X3)
Deionized water	1L	2L	3L
Ammonium chloride (NH ₄ Cl)	26.75 g	53.49 g	80.24 g
<u>Solution 2: Phosphate</u>	<u>Quantity (grams)</u>	Quantity (X2)	Quantity (X3)
Deionized water	1L	2L	3L
Sodium Phosphate. Dibasic, 12-Hydrate, Crystal (Na ₂ HPO ₄ *12H ₂ O)	0.4 g	0.8 g	1.2 g
<u>Solution 3: Iron* (Combine with 4 immediately prior to addition to seawater)</u>	<u>Quantity (grams)</u>	Quantity (X2)	Quantity (X3)
Deionized water	1L	2L	3L
Ferrous sulfate (FeSO ₄ *7H ₂ O)	0.278 g	0.556 g	0.834 g
<u>Solution 4: EDTA *(Combine with 3 immediately prior to addition to seawater)</u>	<u>Quantity (grams)</u>	Quantity (X2)	Quantity (X3)
Deionized water	1L	2L	3L
Disodium Ethylenediamine Tetraacetate (Na ₂ EDTA)	3.72 g	7.44 g	11.16 g
<u>Solution 5: Manganese</u>	<u>Quantity</u>	Quantity (X2)	Quantity (X3)
Deionized water	1L	2L	3L
Manganese Chloride (MnCl ₂)	0.0198 g	0.0396 g	0.0594 g

<u>Solution 6: Vitamins* (Store in freezer)</u>	<u>Quantity</u>	<u>Quantity (X2)</u>	<u>Quantity (X3)</u>
Deionized water	1L	2L	3L
Thiamine	0.2 g	0.4 g	0.6 g
Biotin	0.001 g	0.002 g	0.003 g
Vitamin B12	0.002 g	0.004 g	0.006 g

Notes on VSE preparation:

- **The original source of nitrogen for VSE is sodium nitrate (NaNO₃). This can also be used at 42.5 g per 1 L of deionized water. The original source of phosphate for VSE is Na₂ β-glycerophosphate. This can be substituted at 6.48 g per L of deionized water.**
- Prepare all six solutions separately using clean sterilized volumetric flasks, clean pipettes, digital balance, and mix with magnetic stirring bars.
- Filter sterilize each solution using a 0.2µm filter and a vacuum pump assembly. Vitamins should not be heat sterilized.
- Media solutions should be stored in the refrigerator, vitamins (solution 6) should be stored in the freezer and thawed for use
- All chemicals should be dated when received and when opened on the bottle
- All solutions should be clearly labeled at every step, and aseptic technique should be used in preparation
- It is easiest to make up large amounts of the solutions initially, then aliquot out usable amounts in smaller bottles (well sealed) for convenience. The prepared bottles can then be kept ready in the refrigerator for water changes.
- Full strength is 1ml/L
- Germanium dioxide is another, separate component that is added to cultures to prevent growth of diatoms. The solution can be prepared in advance and refrigerated. This solution is added at 2mL/L of water.
- All glassware should be sterilized, and the working space should be very clean and include a flame of some sort to prevent contamination of this high nutrient media.

Nutrient Media Supplies List

Media preparation	
Glassware: (Pyrex)	
	Volumetric flask (1000mL; measuring)
	Erlenmeyer flasks (3000mL; mixing and storing)
	Graduated cylinders (500 or 1000 mL)
	Storage flasks and bottles
	Digital scale (0.0001 g; 3 decimal places)
	Weigh paper/boats
	Metal chemical spoon or spatula (measuring)
	Safety goggles and gloves
	0.2 micron filter for media sterilization (Corning Disposable Sterile Bottle Top Filter, 150mL Funnel, #25965-45)
	Pyrex screw-cap media storage bottles (media storage)
	Clean work space
	Flame (for sterile technique; Bunsen burner, etc)
	Sterile pipettes, 10mL (measuring; disposable plastic or glass autoclavable)
	Pipette bulb or motor
	pH meter and associated buffers
	Stirring plate and magnetic stir bars
	Parafilm

General Culture System Materials and Estimated Costs		
Item	Base	
<u>Seawater System</u>		
Seawater filters—3-step cartridge system, down to 1 micron		Online, Hardware stores
Filter cartridge housings	40	Pentek Blue
Filter cartridges (20, 5, 1 micron size)	5	Pentek
Seawater Holding Tank System		
500 gallon plastic holding tank	400	Ace Roto-mold
UV Light for seawater sterilization	400	Smart UV Sterilizers/Emperor Aquatics
External water pump to circulate water	200	Marinedepot.com
Round Polyethylene or Fiberglas Tanks, various sizes	200-2000	Aquatic Ecosystems, tank suppliers
Polycarbonate or glass 10L clear autoclavable Carboys	50-200	Laboratory, Homebrewing suppliers
Flasks		Laboratory suppliers
Petri Dishes		
Forceps		
Microscope		
Pasteur pipettes	20 (box of 200)	www.sigmaldrich.com
Temperature and Power Alarm and Auto Dialer	330	Omegaphone. www.omega.com
<u>Lights</u>		
CW-HO Fluorescent Lamps - High Output - Cool White		Greenhouse growers supply
Light Bulbs & Fixtures	200	Lighting Suppliers
Photoperiodic timers	30	Aquarium, Hardware supply
<u>Aeration</u>		
Aquarium aeration pumps	30	Aquarium Supply
Aeration tubing, small diameter, 25'	6	Aquarium Supply
Rigid Aeration tubing for large tanks, large diameter	30	Aquarium, Aquaculture Supply