WORKSHOP:
MICROALGAL CULTURE

HOSTED BY
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JUNE 5-6, 2019

FWC Sen. Kirkpatrick Marine Lab
11350 SW 153rd Court,
Cedar Key, FL 32625

TOPICS (Presentations & Demonstrations)

- Function of Microalgae
- Mass-Culture Strategies
- Nutrition of Bivalves
- Chemical Ecology of Cultures
- Phytoplankton as Bivalve Foods
- Media preparation & Aseptic transfers
- Quantification Methods

GUEST SPEAKER

DR. GARY WIKFORS, DIRECTOR OF THE
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MICROALGAL CULTURE WORSHOP

June 5-6, 2019

FWC Senator Kirkpatrick Marine Lab
11350 SW 153rd Court, Cedar Key, FL

Guest Speaker:

Dr. Gary Wikfors
Director, NOAA Fisheries Milford Laboratory, Milford, CT

AGENDA

Wednesday, June 5, 2019

10:30 - 11:00 AM	Registration
11:00 - 11:30 AM	Welcome and Introductions
11:30 - 12:30 PM	Introduction to Microalgae
12:30 - 1:15 PM	Lunch (on site)
1:15 - 2:15 PM	Function of Microalgae
2:15 - 3:15 PM	Mass-Culture Strategies
3:15 - 3:30 PM	Break
3:30 - 4:30 PM	Nutrition of Bivalves
4:30 - 5:00 PM	Discussion

Thursday, June 6, 2019

8:00 - 10:00 AM	Hands-on Demonstrations: Quantification methods, Aseptic transfers, Media preparation *Meet at UF/IFAS Nature Coast Biological Station,* *552 1st St, Cedar Key, FL*
10:00 - 10:30 AM	Break (head back to FWC Marine Lab)
10:30 - 11:30 PM	Chemical Ecology of Microalgal Cultures
11:30 - 12:30 PM	Natural Phytoplankton as Bivalve Foods
12:30 - 2:00 PM	Box lunch, Q&As, Discussion and Wrap-up

What We Do

The Milford Laboratory, a world leader in aquaculture science, was established at the request of Connecticut's oyster industry to help expand sustainable oyster harvests. Over the lab's illustrious history, NOAA scientists, working closely with industry and academia, have made fundamental contributions to the understanding of shellfish biology and reproduction (see "History," below). Today, the Milford Lab continues to conduct state-of-the-art science that informs management for the sustainable expansion of aquaculture, provides services

Meet Our Staff »

Upcoming Events

Dr. Gary H. Wikfors

Chief, Aquaculture Sustainability Branch / Lab Director
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Education

- Ph.D. Phycology - University of Connecticut
- M.S. Biology - University of Bridgeport CT
- B.S. Biology - University of Maine at Orono

Gary's terminal degree is in Phycology – the study of algae – but he always has worked at the intersection of phytoplankton and the bivalve mollusks -- such as oysters, clams, scallops, and mussels -- that derive their nutrition from phytoplankton. Gary has studied trophic transfer of pollutants from phytoplankton to bivalves, biochemical nutrition of shellfish, and harmful-algal effects upon bivalves. Much of his research has employed a laboratory-based, experimental approach, but he also has been involved in large, multidisciplinary field studies. Gary was an early adopter of flow-cytometry for microalgal applications, and use of this technology has sparked a subsequent interest in cellular immunity in bivalves and other invertebrates. As Chief of the Biotechnology Branch, Gary has a hands-on role in several current team initiatives: 1) Nutrient bioextraction using shellfish aquaculture, 2) Probiotic bacteria for use in shellfish hatcheries, and 3) Shellfish cellular immune response to environmental variation.

ENCYCLOPEDIA OF

AQUACULTURE

MICROALGAL CULTURE

GARY H. WIKFORS
Northeast Fisheries Science Center
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OUTLINE

WHAT ARE MICROALGAE?

By direct translation from Latin, microalgae are "little seaweeds." However, defining microalgae further is not simple, because the microalgae represent a taxonomically diverse group of organisms, rather than a single, phylogenetic category. A functional definition of microalgae might be "photosynthetic single-celled or colonial microorganisms"; however, most of these microbes are able to grow without light if dissolved sugars are provided. Microalgal cells range in size from one micrometer — roughly the size of a bacterium — to several hundred micrometers (1 μm) — barely visible to the naked eye. Colonies and chains of some microalgal cells can attain a length of several centimeters (2.5 cm = 1 in.). This group of organisms contains remarkable morphological diversity, with shapes ranging from simple spheres to the ornate, silica shells of one group, the diatoms. Many microalgae are motile, propelling themselves with flagella, by amoeboid motion, or by gliding on extruded mucilage. Microalgae are found in an astonishing range of habitats, such as in fresh, saline and hypersaline waters, in polar ice, in soil, attached to plants and animals, and even in symbiotic relationships with fungi (e.g., lichens) and animals (e.g., corals). Historically, many of these organisms have been claimed and named by both zoologists and botanists; therefore, taxonomy has been, and remains, problematic. From the perspective of aquaculture, there are common characteristics that warrant their consideration as a functional group; the foregoing definition will suffice for this discussion.

As "microalgae" is a functional rather than phylogenetic group, a list of taxa that would reasonably fit within the category is necessary (see Table 1). Higher level systematics of these groups are under revision; hence, only the class level is specified. The diversity of the group is underscored by the presence of both prokaryotic (not containing a nucleus, Cyanophyceae, or cyanobacteria) and eukaryotic (nucleated) taxa. The eukaryotic groups are thought to have arisen from the incorporation of photosynthetic prokaryotes (or, subsequently, photosynthetic eukaryotes) within protozoan-like host organisms. This hypothesized process is referred to as the endosymbiosis theory. Evidence from both electron microscope studies of microalgal cells (usually focused upon numbers and types of membranes within the cell) and more recent molecular sequencing work indicates that endosymbiotic creation of "new" organisms may have occurred a number of times in evolutionary history, leading to the diversity in morphology and physiology seen today. This diversity, especially in terms of physiology, provides opportunities for the current and potential use of these organisms in the aquaculture industry.

Table 1. List of Currently Recognized "Microalgal" Classes and Representative Genera Used in Aquaculture

Class	Common Name	Representative Genera Used in Aquaculture
Cyanophyceae	Blue-green algae	*Spirulina*
Prochlorophyceae	Prochlorophytes	None
Rhodophyceae	Red algae	*Porphyridium*
Prasinophyceae	Scaled green algae	*Tetraselmis* *Pyramimonas*
Chlorophyceae	Green algae	*Chlorella* *Dunaliella* *Haematococcus*
Cryptophyceae	None	*Cryptomonas* *Rhodomonas*
Chlorarachniophyceae	None	None
Euglenophyceae	None	None
Dinophyceae (Pyrrophyceae)	Dinoflagellates	*Crypthecodinium*
Chrysophyceae	Golden-brown algae	None
Raphidophyceae	None	None
Eustigmatophyceae	None	*Nannochloropsis*
Xanthophyceae (Tribophyceae)	None	None
Bacillariophyceae	Diatoms	*Thalassiosira* *Chaetoceros* *Nitzschia*
Dictyophyceae	None	None
Pelagophyceae	None	None
Haptophyceae (Prymnesiophyceae)	None	*Isochrysis* *Pavlova*

WHY CULTURE MICROALGAE?

There are two main reasons for which microalgae are grown: (*1*) for extractable chemicals and (*2*) as feeds for aquacultured animals. Efforts to produce foods for direct human consumption have met with limited success, and crops such as the cyanobacterium *Spirulina* remain in the

realm of "dietary supplement," rather than food crop; these cases are considered along with extractable chemicals.

Microalgae for Extractable Chemicals

Chemical analyses of microalgae have led to the discovery of many novel chemical compounds, some of which are useful as food additives, pharmaceuticals, cosmetics, or in other high-value applications. Examples of microalgae currently commercially cultured for extractable chemicals include (*1*) *Dunaliella* for β-carotene, a human nutritional supplement; (*2*) *Haematococcus* for the pigments astaxanthin and canthaxanthin, which are used as coloring agents in salmon feeds; (*3*) *Crypthecodinium* for docosahexaenoic acid (DHA), which is incorporated into infant formula; and (*4*) the aforementioned *Spirulina*, which is used as a dietary supplement or additive to cosmetic products. In most cases, the chemical compound of commercial interest is present as a small fraction of the total algal biomass; therefore, the scale of cultures for commercial production generally is in the range of many cubic meters ($1\ m^3 = 35.3\ ft^3$). When a selected portion of the algal biomass is extracted and refined, the presence of chemical and biological contaminants within production cultures is not relevant, unless overall production is constrained or an undesired chemical is coextracted with the product compound. Thus, in many cases microalgae for extractable compounds are cultured in large, open-pond facilities; *Dunaliella* ponds of several hectares (1 ha = 2.4 acres) in size are characteristic. Alternatively, industrial, "brewery-type" technology may be employed, as for *Crypthecodinium* cultured heterotrophically for DHA.

Although the list of successful commercial products that are extracted from cultured microalgae is short at present, the potential is enormous. Natural-products chemists have described numerous novel compounds from microalgae, including chemicals with antibiotic, antitumor, and neuroactive characteristics. As such products are developed for commercialization, considerable growth in microalgal culture for extractable compounds is predicted.

Microalgae as Aquaculture Feeds

The most common reason for culturing microalgae is as a feed in an aquaculture food chain (especially for marine or estuarine animals). Microalgae are consumed directly by bivalve molluscs (e.g., clams mussels, oysters, and scallops) throughout life, by young stages of crustaceans (shrimp), and even by first-feeding stages of some finfish. In addition, microalgae can be used to grow small invertebrates, such as rotifers (*Brachionus* sp.) and brine shrimp (*Artemia* sp.), that are fed to young stages of crustaceans and finfish and are used to enrich these small invertebrates with nutritional compounds, especially fatty acids and sterols, required by the larval crustaceans and finfish.

Criteria for selection of microalgae as aquaculture feeds include ease of culture, size, digestibility, and nutritional value. All but the first of these criteria are covered in another entry of this volume. Ease of culture under particular circumstances will be dependent upon the tolerance of the alga to physical, environmental conditions, such as temperature and salinity. An alga's ability to coexist with or exclude microbial contaminants, ranging from bacteria to fungi and protozoans, also is critically important in most commercial applications.

A number of specific, clonal strains of microalgae have been found, empirically, to possess desired characteristics and are in wide use; these can be obtained from aquaculture supply companies, academic institutions, and government-funded institutions that maintain microalgal culture collections. The strain level of identity is appropriate, because microalgal taxonomy remains in a state of continuing development and because different isolates of the same taxonomic species may differ widely in growth or nutritional characteristics relevant to their use as aquaculture feeds.

HOW DO MICROALGAE WORK?

Energy

Microalgae are referred to as autotrophs, a word that translates literally as self-feeding. This term is wholly appropriate because the process of photosynthesis, by which light energy is converted to chemical energy, provides sugars that are subsequently eaten, or burned, to support the heterotrophic processes of the rest of the cell. In cyanobacteria, photosynthesis occurs in cellular structures, called lamellae, that are not segregated by membranes from the rest of the cell. The photosynthetic apparatus of eukaryotic microalgae is contained within a membrane-bound structure, called a plastid or chloroplast, that is thought to have arisen, evolutionarily, from a cyanobacterial endosymbiont.

Regardless of its location within the cell, the photosynthetic process itself accomplishes nothing more than creating sugars and oxygen from carbon dioxide and water — a transforming process in the natural history of the earth, but insufficient in itself to sustain life. The reverse process, catabolism of sugars to release energy and carbon dioxide, is necessary for the cell to make use of the energy captured within the sugars. This concept is emphasized, because the energy and gas (oxygen and carbon dioxide) dynamics of microalgal cultures, particularly in natural diurnal light cycles, can vary considerably. Heterotrophic processes are active continuously to sustain life, while autotrophic processes occur only in the presence of light. In dim light, or in bright light with self-shading in dense cultures, light may be insufficient to counterbalance heterotrophic processes. The level of light energy input needed to just sustain the population without growth is called the compensation point. For the population of microalgae to increase, light energy above the compensation level must be provided.

Chlorophyll α, the chemical compound that catalyzes photosynthesis, absorbs light in the wavelength range of 400–700 nm, this range is referred to as photosynthetically active radiation, or PAR. Full, noon sunlight is in the range of 2,000 μmol photons per square meter per second of PAR; other units of PAR flux encountered may be micro-Einsteins per square meter per second, shortened by microalgal icon Ralph Lewin to Alberts. Thus, both

the quantity and quality of light is important to energy acquisition by microalgae.

An often-overlooked aspect of the energy-input requirements of microalgae is the potential to supply sugars to the heterotrophic cellular machinery from a source other than photosynthesis. Indeed, most microalgae tested are physiologically able to grow on sugars added to the culture medium as the sole energy source (i.e., in the dark). This capability has only recently been exploited commercially, and only under highly controlled, bacteria-free conditions. Bacterial sugar uptake and growth rates generally are more rapid than those of microalgae. In a competition for dissolved sugars, bacteria have an advantage over microalgae; therefore, aseptic production is necessary. In addition, while it may be more cost-effective to provide artificial sugars than artificial light to algae, the biochemical composition of microalgae grown photo-autotrophically vs. heterotrophically may affect the ultimate cost–benefit decision.

Materials, or Nutrients

Life processes require two inputs: energy and materials. The energy needs of the microalgae just discussed interact with materials — either carbon dioxide and water, or sugars and oxygen — and the material needs, predictably, interact with energy status of microalgal cells as well. At least 24 chemical elements have been identified as being essential, (i.e., nutrients) in all living cells (see Table 2). Although all of these elements could pass through membranes surrounding cells by simple diffusion, active, energy-consuming uptake processes have been demonstrated for essentially all nutrients. Beyond the simple fact that diffusion would be highly rate limiting with dilute, natural nutrient concentrations, many required elements exist as chemical compounds or complexes in solution that must be modified before the element can be assimilated by the cell. Thus, nutrient uptake can be considered an active, energy-requiring process.

Table 2. Chemical Elements Considered to be "Essential" for Living Cells, Including Microalgae

Element	Chemical Symbol	Typical Source in Microalgal Culture Media
Carbon	C	Carbon dioxide in air
Hydrogen	H	Water
Oxygen	O	Water
Nitrogen	N	Nitrate, ammonia, urea
Phosphorus	P	Phosphate salts
Calcium	Ca	Calcium carbonate
Sodium	Na	Sodium chloride
Chlorine	Cl	Sodium chloride
Magnesium	Mg	Magnesium chloride or sulfate
Potassium	K	Potassium chloride
Sulfur	S	Sulfate salts
Boron	B	Boric acid
Iron	Fe	Ferric chloride
Selenium	Se	Selenous acid
Copper	Cu	Cupric chloride or sulfate
Manganese	Mn	Manganese chloride
Zinc	Zn	Zinc chloride or sulfate
Molybdenum	Mb	Molybdenum chloride
Cobalt	Co	Cobalt chloride or vitamin B_{12}
Iodine	I	Potassium iodide
Nickel	Ni	Nickel chloride
Silicon	Si	Sodium silicate
Fluorine	Fl	Sodium fluoride
Chromium	Cr	Dichromate salts

Microalgal nutrients can be segregated into two arbitrary categories: macronutrients and micronutrinents, or trace elements. These categories are arbitrary in that deficiency in any element, macro or micro, will constrain growth. For all microalgae, nitrogen (N) and phosphorus (P) are considered macronutrients and are major structural components of proteins, nucleic acids, and the energy-management chemicals (e.g., adenosine triphosphate, or ATP) of the cells. For one class of microalgae, Bacillariophyceae or the diatoms, silica (Si) also is a macronutrient, because it is required in relatively large amounts for the formation of cell walls.

Forms of nitrogen biologically available to microalgae include ammonium (all), nitrate (some cannot use), and organic compounds (urea, amino acids, etc., available to most microalgae). Ammonium is the nitrogen "currency" within the cell, while nitrate must be reduced and urea catabolized during uptake; therefore, thermodynamics favors ammonium uptake. Most commonly used microalgal nutrient enrichments provide nitrate, though, because it is more stable in solution (not volatile) and not available to many bacteria in contaminated cultures. Microalgae that are not able to produce nitrate reductase, the enzyme that catalyzes the reduction of nitrate to ammonium, generally must be grown on ammonium. As nitrogen is a major component of proteins, deficiency arrests protein synthesis and may lead to an accumulation of energy-storage products, such as starches and lipids, in microalgal cells; this response can be exploited if increased storage-product yield is desired in cultures.

Phosphorus exists in natural waters chiefly in the oxidized form phosphate, although some organic phosphorus compounds may exist as well. Phosphate is by far the most common form of phosphorus added to microalgal culture media, and amounts added generally are in the range of one phosphorus atom for every 16–25 nitrogen atoms. Phosphorus deficiency in algal cells may lead to physiological disruption of protein synthesis, similar to nitrogen deficiency, but also may disrupt energy management within the cell; therefore, phosphorus starvation is a less dependable culture-management strategy than nitrogen starvation.

As previously mentioned, silica is required for the formation of cell walls in diatoms. As for nitrogen and phosphorus, an oxidized form, silicate, is encountered in natural waters and added to culture media. Different diatoms require different amounts of silica, and most possess the ability to make thinner "shells" under silica deficiency. Extreme silica deficiency arrests cell division and may cause cells to accumulate storage products, providing a possible culture-management strategy.

Trace elements, or micronutrients, represent a group of elements for which cellular needs are several orders of magnitude lower than the macronutrients. Micronutrients

tend to be minor but necessary components of enzymes involved in basic cell processes. Many of these elements are metals that are present in natural, oxygenated waters in highly oxidized forms, often complexed with dissolved organic molecules. In these forms, limited ability of microalgal uptake mechanisms to remove free ions from stable complexes may limit bioavailability and growth. In culture media, artificial complexing, or chelating, agents, such as EDTA, NTA, or citric acid, are added to moderate the bioavailability of trace metals, in particular. When chelating agents are used, metals must be added in excess of microalgal needs to saturate chelator-metal complex sites; thus, levels of trace metals added to media with artificial chelators may far exceed levels that would be toxic if metals were in the free-ion form. Indeed, the concentration range for many metals between deficiency and toxicity is relatively narrow; therefore, trace-element management of microalgal cultures can be quite challenging.

Some microalgae used widely in aquaculture (e.g., the prymnesiophytes) require vitamins, usually B_{12} and thiamin, and sometimes biotin. In open, bacterized cultures, bacteria generally supply these growth factors; however, pure cultures require that these vitamins be added in trace quantities for the algae that require them.

Microalgal Growth = Population Growth

If suitable and sufficient energy and materials are provided to an algal cell, it divides into two cells, which separate and become distinct individual organisms. Hence, microalgal growth is characterized in terms of increases in numbers of individual cells, rather than in terms of increases in an individual's size, which is the case for metazoan organisms. Production rate of microalgal biomass in a culture often is quantified in terms of average number of cell divisions per unit time (divisions per day), or the reciprocal, average time between cell divisions. A mathematical description of progressive cell divisions is a logarithmic function; calculation of this function from cell counts at two or more times is a straightforward procedure and a powerful tool for describing and managing a culture's performance.

There are two fundamental management strategies for microalgal cultures: batch culture and continuous culture. In a batch culture, a small population of cells (inoculum) is placed within a relatively large container supplied with sufficient energy and materials to produce a much larger number of cells. The production rate and the maximum number of cells that can be supported in the large container can be constrained by either energy or materials, but the main point is that algal growth will stop eventually, when there is no longer sufficient energy or materials to support further cell divisions. Yield and time of active growth are finite in a batch culture, and the container must be cleaned and reinoculated at the end of each growth cycle. In continuous culture, materials and energy are supplied continuously, and cells are removed at a constant rate equal to, or less than, the rate of production of new cells. Thus, a continuous culture has an indefinite lifespan, as long as the production of new cells is at least as fast as the removal of cells. There are a number of different categories of continuous cultures; the term chemostat, describing but one type of continuous culture, often is misapplied to cultures with constant fluid replacement rates (cyclostats), or constant standing biomass (turbidostats).

In theory, it would appear that continuous culture offers overwhelming advantages over batch culture, in terms of effort (labor), production-rate optimization, and management and control. In practice, however, it can be very difficult to maintain the steady-state conditions necessary for most continuous cultures. Furthermore, living contaminants, such as bacteria and protozoans, can divide more quickly than the cultivated alga, eventually replacing the intended crop. A compromise between batch and continuous culture management strategies, semicontinuous culture, offers some advantages of both. A single culture can be maintained for an extended period of time, replenishment rate (both amount and time between partial harvests) can be varied in response to culture performance, and partial harvests can be delayed to allow accumulation of lipids or carbohydrates if these are important in the application. Recent efforts to incorporate contemporary, computer process-control technology into microalgal culture have improved the effectiveness of continuous and semicontinuous microalgal culture methods.

HOW ARE MICROALGAE CULTURED?

Containers

Essentially, any vessel or structure that can contain water can be used to culture microalgae. Experimental and "seed" cultures routinely are grown in test tubes, flasks, jars, bottles, etc., made of glass and various kinds of plastic. Tanks, tubes, buckets, barrels, bags, pools, and ponds, constructed of nearly limitless materials, have been applied to production-scale microalgal cultures. For photosynthetic production, light can be provided from any direction if the container is transparent to light in the PAR range. Opaque containers with the artificial light sources immersed within the culture have been used with limited success. Alternatively, containers with an exposed surface lighted from above can have opaque sides and bottom. In addition to permitting light penetration, containers must provide for gas exchange with the atmosphere or incorporate an added gas stream. Tank and pond cultures generally are kept relatively shallow to provide for gas exchange with the atmosphere, by virtue of a high surface-to-volume ratio, and also to maintain light input above the compensation level. Gas exchange and culture mixing often are enhanced by introduction of a diffused gas stream (bubbles) through the culture. Although widely used, bubbling of open, bacterized microalgal cultures may encourage bacterial degradation of the culture; physical mixing with foils, paddles, etc., may be more successful and has been applied in many large systems. Tubular containers, consisting of many meters of narrow-diameter, transparent glass or plastic tubing have seen several periods of intense interest, as this design offers some advantages of both open containers (short light path) and closed systems

(exclusion of contaminants). Gas-transfer limitations have constrained effectiveness of tubular systems. For heterotrophic microalgal production, standard industrial bioreactors used for other microorganisms have proven to be transferrable, with few modifications, to most microalgae.

Energy

Most commercial microalgal production is based upon photosynthetic growth. Light can be provided by natural sunlight or by artificial lights. Natural sunlight is inexpensive (a function of land cost), but varies seasonally and may be unreliable daily, depending upon location and climate. Artificial lights can be controlled very precisely, in terms of both quantity and quality, but can account for up to 95% of the cost of culturing microalgae. Many small-scale fish and shellfish hatcheries culture microalgal feeds using artificial lights because feed production must coincide reliably with seasonal life-history stages of the animals that are, in turn, often nonsyncronous with seasonal light and temperature cycles. Accordingly, the cost of producing microalgal feed cultures in these facilities is among the highest of all cultivated products, ranging from $100 up to $400 per dry kg ($45 to $180 lb) of microalgal biomass. In contrast, culture of *Dunaliella* for β-carotene in desert ponds can cost over 100 times less, because of the difference in energy cost alone. For heterotrophic culture, various waste and off-specification agricultural products can be used as energy sources for microalgal culture. The economics of using reduced-carbon energy sources for heterotrophic production appear to be intermediate between artificial light and solar energy; the success of each type of energy source is dependent upon the application of the microalgal product and the specific alga that is cultured.

Materials

Fertilization of microalgal cultures ranges, in commercial practice, from animal manure added directly to earthen ponds to complex formulations prepared with pharmaceutical care. In instances where extractable chemicals are the product, industrial-grade chemicals are generally sufficient, because chemical contamination of the product is not a practical concern. When cultured microalgae are part of a food chain leading to a human food product, more refined chemical fertilizers are recommended. Culture medium formulations can be assembled from component chemicals according to recipes found in standard texts (see Bibliography), but proven microalgal fertilizer mixtures, based upon the Guillard "f/2" formulation, now are being marketed by aquaculture-supply companies.

Culture Management

Batch culture still is used in most applications. Usually, small-scale flask cultures maintained under pure (bacteria-free) conditions are increased in volume by inoculating a series of progressively larger containers until the production-scale is reached; the term for this practice is "progressive batch culture." For many aquacultured animals fed microalgal cultures, the lipid content of the algal feed is the most critical nutritionally; therefore, many farmers allow cultures to enter a nutrient-deficient (usually nitrogen) phase, during which the nondividing microalgal cells often store photosynthetic energy as lipid. Continuous and semicontinuous culture methods are employed routinely in extractable-product operations and increasingly are being developed for marine-animal feeds applications.

WHAT INNOVATIONS ARE EXPECTED?

In reviewing the history of the development of modern microalgal culture, one is struck by the existence of two, nearly independent "heritages." One line of investigation, dominated by engineers and chemists, was motivated by the potential for industrial products, ranging from human foods to synthetic fuels and oxygen factories for extended space travel. Working almost exclusively with the freshwater chlorophyte, *Chlorella*, scientists and engineers developed and built pilot plants employing state-of-the-art electronics and fluidics innovations. While most of the perceived applications for *Chlorella* biomass have not proved successful, the legacy of this research effort provided the foundation for today's extractable-product microalgal technology. The second line of investigation, dominated by biologists and ecologists, arose from laboratory-scale culture and feeding apparatus designed to maintain experimental animals for investigation of their life cycles, feeding habits, etc. These types of methods were generalized and modified to culture a wide variety of microalgal types and were used in the elucidation of nutritional requirements of animals that were to subsequently enter into aquaculture production. Little thought was given, however, to economics or problems of scaling the design for commercial use. At the time of this writing, it appears that the two "lineages" of microalgal culturists finally are communicating and collaborating on engineered systems that satisfy the biological needs of the variety of microalgae used as aquaculture feeds, as well as for extractable compounds, and do so in a cost-effective way. This cooperation holds great promise for the future of microalgae in aquaculture.

BIBLIOGRAPHY

Author's Note: The following references were selected to provide background in general principles pertaining to microalgal culture, not as specific how-to manuals. What manuals have been produced, usually by academic or government research laboratories, tend to be narrow in application and availability, and all still require a firm grounding in basic principles to be useful.

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MICROBOUND FEEDS

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OUTLINE

INTRODUCTION

Microbound feed is a general category of small particulate feeds ranging in size from 50 to 700 μm that are typically fed to larval stages of fish and invertebrates. The defining characteristic of these feeds is that the particles are held together by an internal binder, which may be a complex carbohydrate or a protein having adsorptive and adhesive properties (1). This differs from microencapsulated feeds, which are characterized by a distinct wall or capsule surrounding a central core of material (2,3).

There are many types of larval feeds available on the market and more are being developed in laboratories around the world. The particular feeding situation (e.g., species, water temperature, or culture system) determines which type of larval feed to use. Different feeding situations may require different types of feed. To understand the processing methods used in manufacturing microbound feeds, an evaluation of the desired characteristics of an effective feed is necessary.

Palatability, nutrient stability, nutrient availability, and particle stability are all important traits of a microbound feed. A feed of outstanding nutritional quality is of little value if it has low palatability and the animal will not consume it. Palatability is affected by factors such as smell, flavor, and texture. The smell and flavor of the feed is caused by the leaching of nutrients into the water, and thus some leaching is necessary for adequate levels of feed consumption. Excessive leaching, however, can result in a poor quality feed due to reduced nutrient content. Nutrient stability is very important to ensure adequate nutrition, but if the feed is bound too tightly, consumption and/or nutrient absorption could decrease. Feeds for finfish must be formulated, manufactured, and selected with the goals of high palatability and high nutrient stability in mind, even though those objectives seem to work in opposite directions.

Another important characteristic of a microbound feed is particle stability. This refers to the loss of material from the feed particle in water. A feed with low particle stability will disintegrate and degrade water quality, which can decrease survival of the cultured animal. Gill damage caused by high levels of particulate matter in the tank, bacterial contamination, and low oxygen levels are all problems that can be caused by a feed with low particle stability (4).

Feed characteristics are strongly affected by the type of binders and processing methods used in manufacture, so each feeding situation may best be accommodated by a particular formulation or processing method. There can also be interactions between processing method and ingredient formula, including binder source. Sometimes a particular processing method is categorized as ineffective for use in a particular situation, when the formulation itself in combination with the processing method was actually inadequate. The culture technique used when feeding formulated feeds can also determine if the program is a success or a failure. Many factors need to be considered when selecting a larval feed, but by considering formulation, processing method, palatability, nutrient stability, particle stability, and culture methods, an effective larval production program using microbound feeds can be achieved.

CLASSES OF MICROBOUND FEEDS

Microbound feeds can be separated into three major classes according to production process: crumbled, on-size, and complex particles. Crumbled feeds are produced by manufacturing a pellet, flake, or cake that is fractured into smaller pieces and sifted to obtain the desired size (5,6). On-size feeds are manufactured directly to the correct size particle (2,7), which not only saves a production step, but produces physical characteristics that differ from crumbled feeds. Complex particles

Scoresheet for microalgal strains commonly used as aquaculture feeds: biochemical composition.

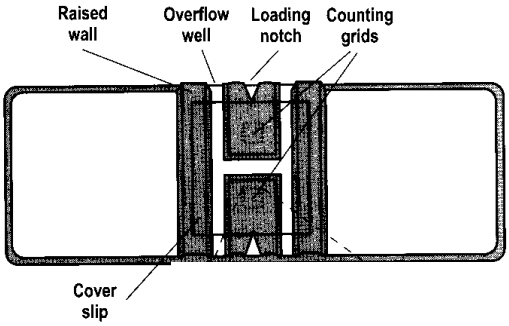
Strain	Genus	Species	EPA	DHA	ARA	Lipid	Starch	Cholesterol	24-Meth Sterols
T-ISO	*Tisochrysis*	*lutea*	0	+	+	+	-	0	0
C-ISO	*Tisochrysis*	*lutea*	0	+	+	+	-	0	0
ISO	*Isochrysis*	*galbana*	0	+	++	+	-	0	0
CCMP-609	*Pavlova*	*sp.*	0	++	++	++	-	0	0
CCMP-459	*Pavlova*	*pinguis*	0	++	++	++	-	0	0
MONO	*Pavlova*	*lutheri*	0	+	++	+	-	0	0
#93	*Pavlova*	*gyrans*	0	+	++	+	-	0	0
Skel	*Skeletonema*	*costatum*	+	+	-	+	+	+	+
3H	*Thalassiosira*	*pseudonana*	+	+	+	+	+	+	+
TW	*Thalassiosira*	*weissflogii*	+	+	+	+	+	+	+
Chaet-cal	*Chaetoceros*	*calcitrans*	+	++	+	+	+	+	+
CHGRA	*Chaetoceros*	*muelleri*	+	++	+	+	+	++	+
Chaet-B	*Chaetoceros*	*neogracile*	+	++	+	+	+	++	+
PLY-429	*Tetraselmis*	*chui*	++	0	+	++	++	0	++
PLAT-P	*Tetraselmis*	*striata*	++	0	+	++	++	0	++
MC:2	*Tetraselmis*	*sp.*	++	0	+	++	++	0	++
Rhodo	*Rhodomonas*	*(lens)*	0	+	+	+	+	+	0
3C	*Cryptomonas*	*salina*	0	+	+	+	-	+	0

Scoresheet for microalgal strains commonly used as aquaculture feeds: "culturability" in containers.

Strain	Genus	Species	Carboy	Kalwall	Bag	Tank
T-ISO	*Tisochrysis*	*lutea*	++	+	+	+
C-ISO	*Tisochrysis*	*lutea*	++	+	+	+
ISO	*Isochrysis*	*galbana*	++	+	+	+
CCMP-609	*Pavlova*	*sp.*	+	-	-	-
CCMP-459	*Pavlova*	*pinguis*	+	-	-	-
MONO	*Pavlova*	*lutheri*	+	-	-	-
#93	*Pavlova*	*gyrans*	+	+		+
Skel	*Skeletonema*	*costatum*	++	+	+	+
3H	*Thalassiosira*	*pseudonana*	+	-	+	+
TW	*Thalassiosira*	*weissflogii*	++	+	+	++
Chaet-cal	*Chaetoceros*	*calcitrans*	++	++	+	+
CHGRA	*Chaetoceros*	*muelleri*	++	++	+	+
Chaet-B	*Chaetoceros*	*neogracile*	++	++	+	+
PLY-429	*Tetraselmis*	*chui*	++	-	-	++
PLAT-P	*Tetraselmis*	*striata*	++	-	-	++
MC:2	*Tetraselmis*	*sp.*	++	-	-	++
Rhodo	*Rhodomonas*	*(lens)*	-	-	-	+
3C	*Cryptomonas*	*salina*	-	-	-	

Preferred culture management, based upon practical experience at largest scale.

Strain	Genus	Species	Container	Batch	Semi-continuous	Continuous
T-ISO	*Tisochrysis*	*lutea*	Kalwall	3	1	2
C-ISO	*Tisochrysis*	*lutea*	Kalwall	3	1	2
ISO	*Isochrysis*	*galbana*	Kalwall	1	2	3
CCMP-609	*Pavlova*	*sp.*	Carboy	1	2	3
CCMP-459	*Pavlova*	*pinguis*	Carboy	1	2	3
MONO	*Pavlova*	*lutheri*	Carboy	3	1	2
#93	*Pavlova*	*gyrans*	Carboy	3	1	2
Skel	*Skeletonema*	*costatum*	Kalwall	3	1	2
3H	*Thalassiosira*	*pseudonana*	Kalwall	1	2	3
TW	*Thalassiosira*	*weissflogii*	Kalwall	3	1	2
Chaet-cal	*Chaetoceros*	*calcitrans*	Kalwall	3	1	2
CHGRA	*Chaetoceros*	*muelleri*	Kalwall	3	1	2
Chaet-B	*Chaetoceros*	*neogracile*	Kalwall	3	1	2
PLY-429	*Tetraselmis*	*chui*	Tank	3	1	2
PLAT-P	*Tetraselmis*	*striata*	Tank	3	1	2
MC:2	*Tetraselmis*	*sp.*	Tank	3	1	2
Rhodo	*Rhodomonas*	*(lens)*	Carboy	3	1	2
3C	*Cryptomonas*	*salina*	Carboy	3	1	2



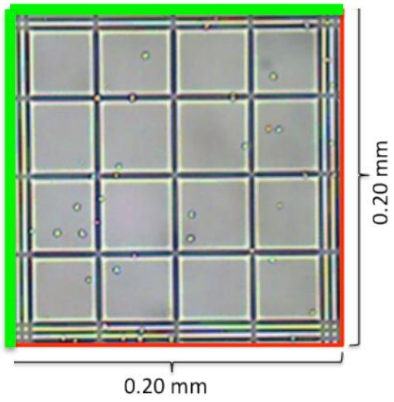
Counting Algal Cells Using a Hemocytometer

Reference: Hadley, N. H., et al. "A Manual for the Culture of the Hard Clam *Mercenaria spp*. in South Carolina." SC Sea Grant Publ. (1997).

Description: The slide is divided into 5 counting areas: 4 corner regions and 1 central region, each of which contain exactly 0.0001 ml of sample. These counting regions are subdivided: 16 small squares in the corner regions and 25 small squares in the central region.

Procedure:

1. If counting motile algal species, add 1-2 drops of alcohol to algal sample.
2. Place coverslip on hemocytometer.
3. Mix sample for 30 seconds.
4. Using a pipette add sample to hemocytometer chamber. Allow the sample to be sucked into the void until the chamber is filled. Do not overfill.
5. Wait a few minutes to ensure all algal cells settle down.



Note for counting algal cells on square border:
Only count the algal cells touching the top and left sides of the square (shown in green). Do not count the cells on the right and bottom sides of the square (shown in red).

Counting Options and Calculations:

A. Count algal cells in all **25 small squares in the central region.**

$$Count \times 10{,}000 = cells/mL\ in\ algal\ sample$$

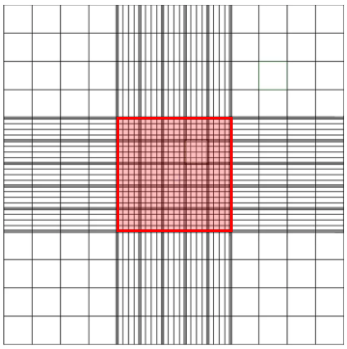
B. If concentration is high, a subsample of the smaller squares may be counted. Count algal cells in any **5 of the 25 small squares** in the central region. Example shows counts done on 5 small squares on a diagonal.

$$Count \times 5 \times 10{,}000 = cells/mL\ in\ algal\ sample$$

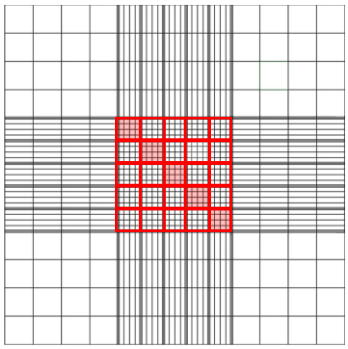
C. When algal concentration is low (<500,000 cells/mL), count algal cells in **all five regions**.

$$Count \times 2{,}000 = cells/mL\ in\ algal\ sample$$

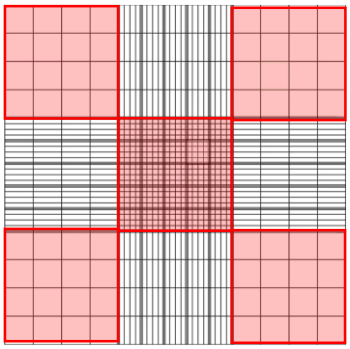
A

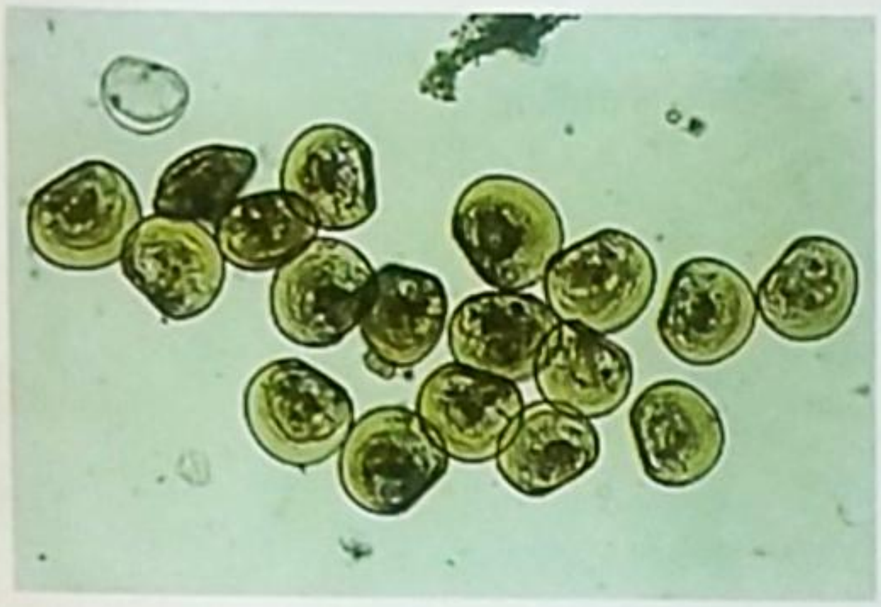


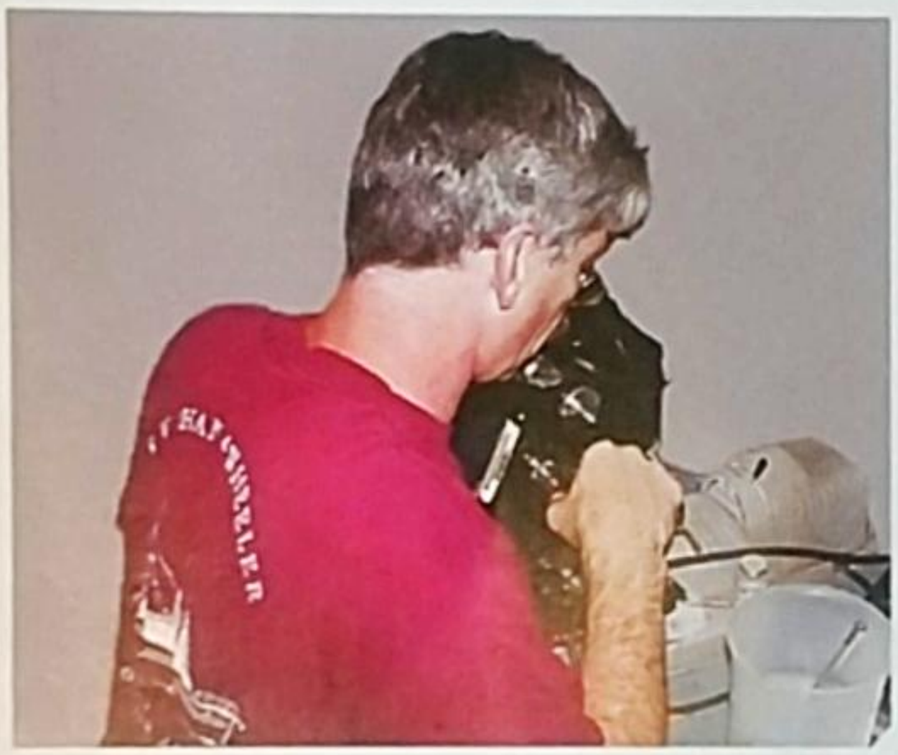
B

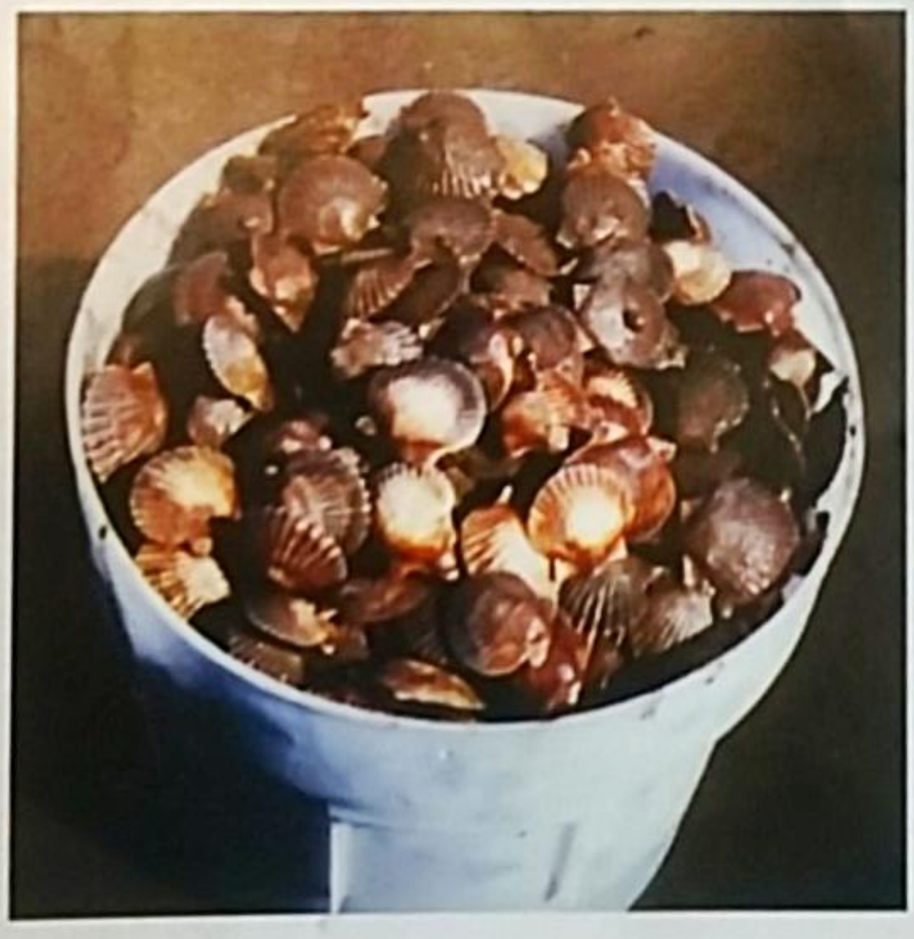


C









Manual for Hatchery Culture of the Bay Scallop

National Marine Fisheries Service
Northeast Fisheries Science Center
Milford Laboratory
Milford, Connecticut 06460

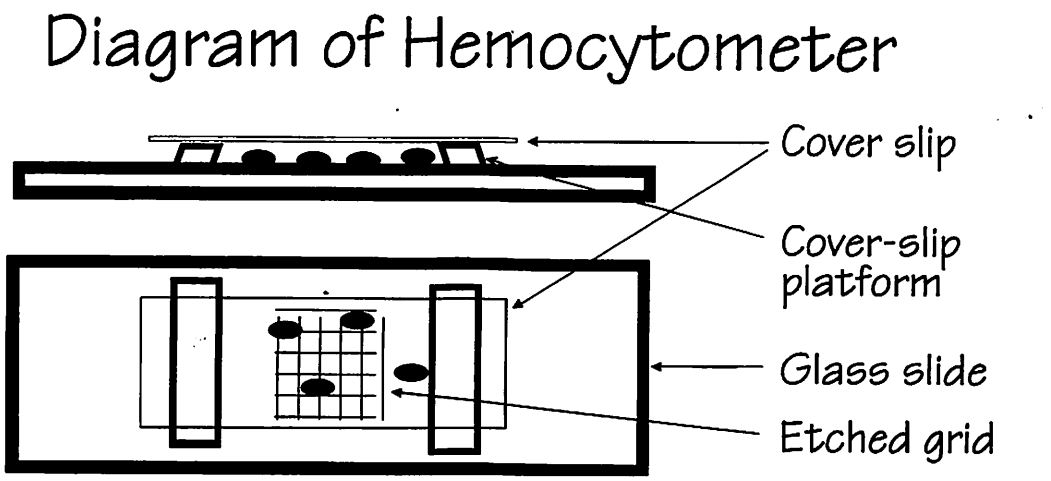
Sea Grant
Connecticut

APPENDIX A

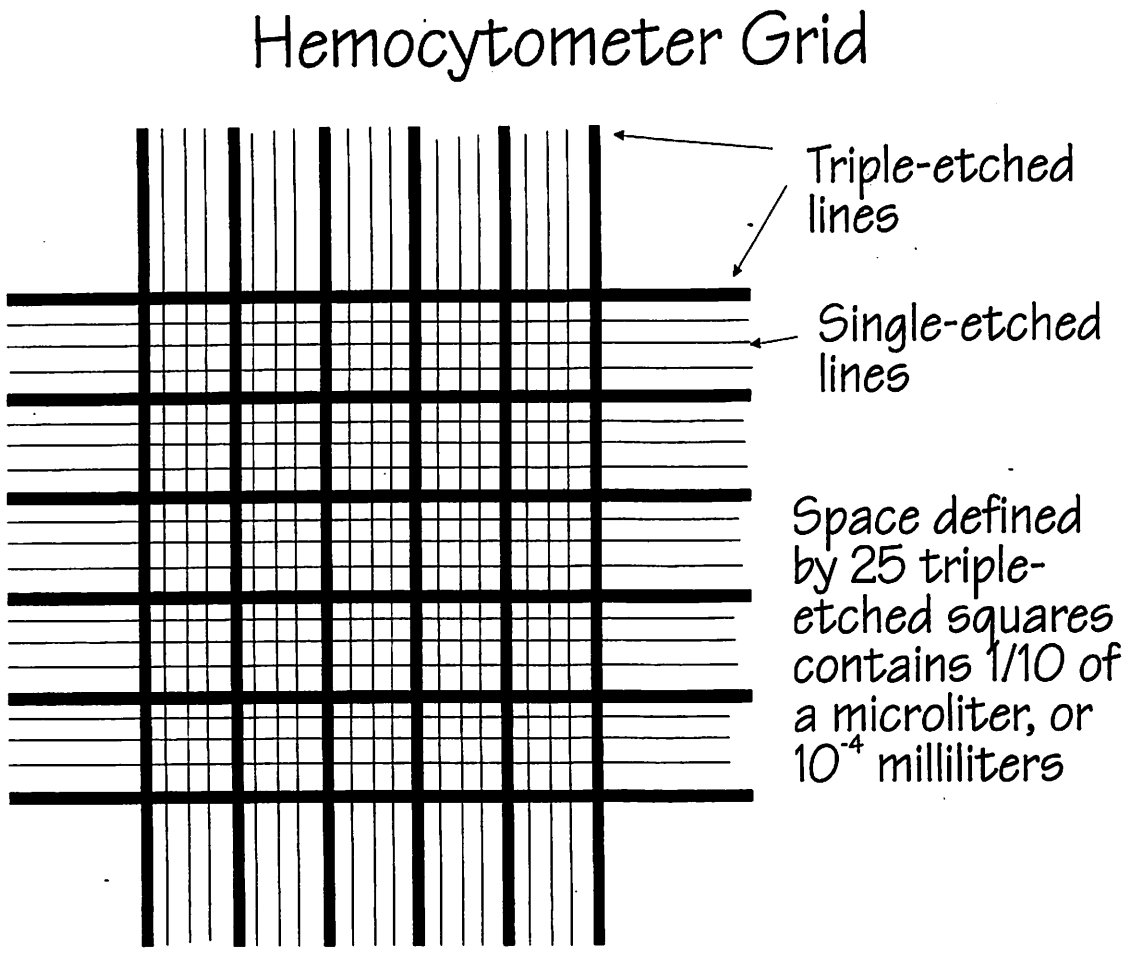
QUANTIFYING MICROALGAL FEEDS

Unlike cells of metazoan plants and animals, microalgal cells separate from each other after dividing; therefore, as algal cultures "grow," you get more rather than bigger algae. For this reason, microalgal feeding rates for scallops must take into account the volume of algal culture fed to a population of scallops, how many cells are in that volume, and also how big cells of the algal species are. Three practical methods have been developed for quantifying how much algal food is in a given volume of culture: 1) microscope cell counts using a hemocytometer; 2) determination of packed-cell volume in a centrifuged, hematocrit-type tube; and 3) percent transmittance of light through a culture measured with a colorimeter/spectrophotometer. All three of these methods require collection of a sub-sample from the culture and determination of the number of cells in that sub-sample; therefore, it is critical that the culture be mixed thoroughly before sampling so that the subsample measured will be representative of the whole culture. Information provided in this manual facilitates use of cell counts or packed-cell volume to adjust feeding rates on a daily basis.

1) Cell Counting: a hemocytometer is a microscope slide designed for clinical counting of human blood cells. The principle of the hemocytometer is to define a three-dimensional space (volume) in which individual cells can be counted with a microscope; this volume is defined by a cover slip resting upon a platform above a polished glass surface with an etched grid. The etched grid defines the portion of the slide that is counted, and the cover slip limits the "depth" of water above the grid. A subsample is removed from the culture to be counted, killed with a small volume of formalin, Lugol's iodine solution, or iodine crystals (which we use so that dilution by fixing solution need not be calculated), and loaded by pipette into the hemocytometer. A compound light microscope is used to count cells at 100-400X total magnification.



In the Improved Neubauer hemocytometer design that we use, a grid of 25 squares delineated by triple-etched lines contains 10 mL; each of these 25 squares is further subdivided by single lines into 16 squares to facilitate counting at higher magnifications. If all cells within the 25 squares are counted, then the

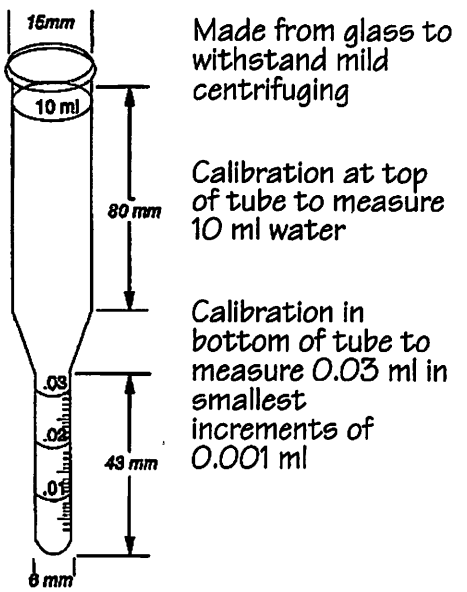


count is multiplied by 10,000 (10^4) to obtain an estimate of the number of cells in one milliliter. In dense cultures, hundreds or thousands of cells may be present in 10^{-4} mL; in these cases, five of the 25 squares are counted and the count is multiplied by 5×10^4 to obtain the number of cells per milliliter. Counts between 30 and 300 recorded with a hand-held counter are considered valid. Advantages of this method include a high degree of precision and accuracy, forced microscopic evaluation of the culture for contaminants, and the ability to compare the culture with much of the published literature which generally reports algal densities in terms of cell number. The main disadvantages of counting cells are that the method is labor intensive (ca. 10 minutes per count), it requires a compound microscope, and hemocytometers are fragile and expensive to replace. Regardless of the method selected for daily quantification of algal feeds, it would be to the advantage of a hatchery operator to be able to do cell counts.

2) **Packed-cell volume (pcv)**: when scallops feed on a relatively dilute suspension of microalgal cells in seawater, they essentially concentrate the cells ingested from the volume of water "filtered" to the volume of their digestive systems. Bivalve larvae will feed actively until their guts are full, spend some time digesting the "meal," expel undigested parts of the microalgae as feces, and then begin feeding again. Thus, the parameter that really defines how much a larva will eat is the volume of cells that it can pack

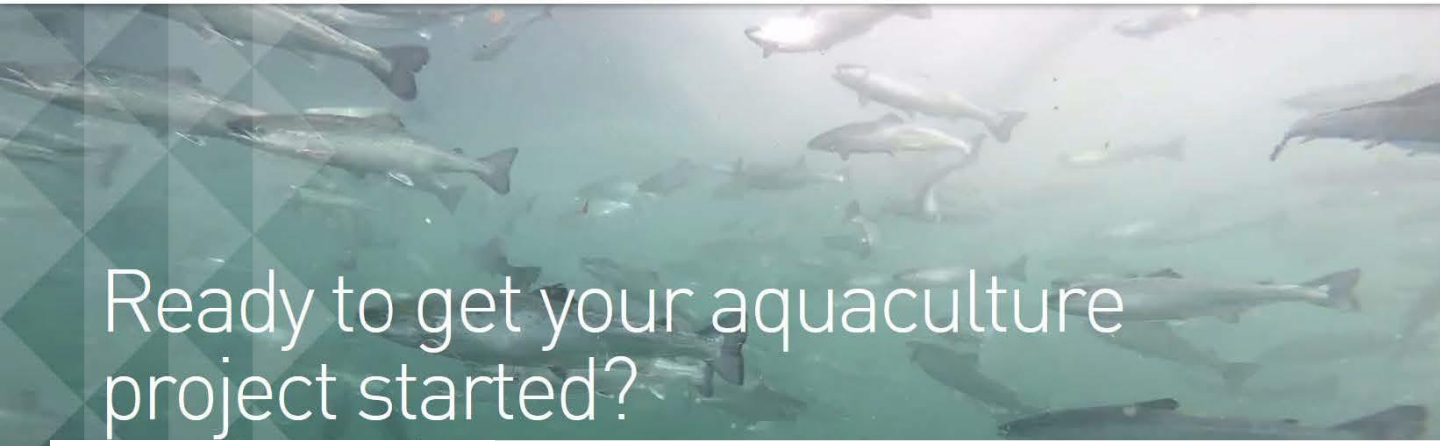
into its digestive system. Measuring packed-cell volume (pcv), which we define as mL of packed algal cells per 10 mL of algal culture, is the method that is most consistent with this view of its food. We employ a centrifuge tube developed to measure the volume of blood cells within a blood sample. This tube consists of a thin, capillary section with etched graduations attached to the bottom of a larger tube that holds 10 mL total volume.

Modified Hopkins tube, after Ukeles, 1973, for determining packed-cell volumes of phytoplankton cultures



10-mL of algal suspension is measured into the tube, and the tube is centrifuged (600XG for 5 min.) so that all algal cells are "packed" into the capillary portion of the tube. The volume of cells, distinct in appearance from the now-clear medium, is read from the scale on the capillary portion of the tube. Packed algal cells are washed from the capillary using a wide-bore needle and syringe, and the tube is re-used indefinitely. Advantages of this method include the aforementioned relevance to the scallops, low labor as six or more samples can be centrifuged and read in less than 10 minutes, and an "automatic" adjustment for cell-size differences between algal strains. Disadvantages include the high cost of pcv tubes (which are no longer in medical use and must be custom-made), need for a centrifuge (although a small, inexpensive, table-top unit will do), and the inability of the method to differentiate between the alga being cultured and any particulate contaminants that may be present in the culture. We use packed cell volume measurements to adjust daily feeding rates for both larval and post-set scallops at Milford, chiefly because of the low labor required and the ability to equalize rations of algal strains of different size.

3) Spectrophotometer/colorimeter: it is easy to see that a culture containing more cells is "darker" than a less dense culture; light coming through a culture is absorbed and scattered by the algal cells in suspension. A spectrophotometer or colorimeter is an analytical instrument that measures the amount of light (usually a defined wavelength range) that passes through a cuvette (a specialized test tube). The simplicity of this principle is seductive, but practicality of application to production feed cultures tempers attractiveness of the method. Calibration of the instrument, calculation of useful values from numbers read on the instrument, and very high potential for interference from contaminants are drawbacks that make this method of quantifying algal-feed production cultures on a daily basis the least suitable. Those wishing to pursue this method further should consult Volume 1 of the *Handbook of Phycological Methods* (Stein, 1973) for detailed procedures.



Part A

ProLine® F/2 Algae Food, Part A, 1 gallon #2398(Qty: 1

Part B

ProLine® F/2 Algae Food, Part B, 1 gallon #2398(Qty: 1

Sodium Metasilicate

Sodium Metasilicate, 1 kg #239804 (In stock) +$ Qty: 1

Price as configured: $71.91

PRODUCT DESCRIPTION | PRODUCT REVIEWS | ADDITIONAL DOCUMENTS

ADD TO CART

- For intensive algae culture in fresh or marine water.
- Vitamin and mineral content ratios equal to Guillard's 1975 F/2 formulation.
- Professional research concentration.
- Complete algae growth media.

Larval fish, penaid shrimp, Artemia species, freshwater prawns, bivalve mollusks and other inverts require algae. Mass cultivation of algae requires a mixture of various vitamins and minerals for maximum algae growth. ProLine F/2 Algae Food provides these essentials in a complete, easy-to-use liquid form. When used as directed, allows fast growth rates and high densities to be achieved. One gallon each of solutions A and B will produce 7,680 gallons of growth media. For culturing diatomaceous organisms, sodium metasilicate must be added to the F/2 Formula.

Topics »

The "What Do Clams Eat" guide assists farmers in identifying potential food sources for hard clams, the spatial and seasonal distribution of food on Florida's east and west coast, as well as whether the food is good (nutritious) or bad (noxious/ harmful) for the clams.

The guide covers topics including sampling methods, food quantity, food quality, and fact sheets on major algal groups

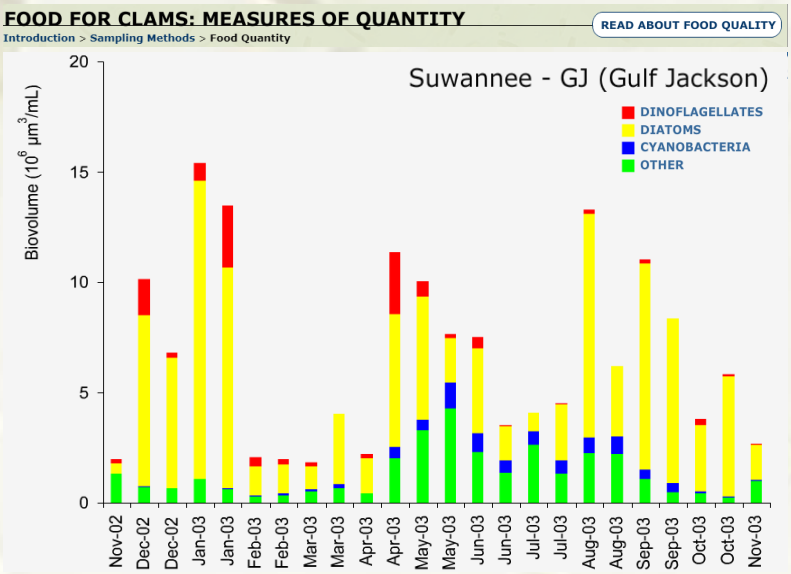
To access the guide, visit shellfish.ifas.ufl.edu and click on the "What Do Clams Eat?" tab. Flash player needs to run.

What do Clams Eat?

Farming of hard clams supports many small businesses in Florida with over 150 million clams produced each year. Farms are located on submerged lands in inshore coastal waters leased from the State of Florida. Florida's warm climate supports year-round productivity and optimal growing conditions for hard clams. Knowledge of what clams eat and when those food items are most available can guide farmers in deciding when to plant seed and harvest clams. In addition, information on the patterns ofoccurrence of harmful algal blooms, such as toxic red tides, can help farmers avoid or anticipate losses in clam stocks.

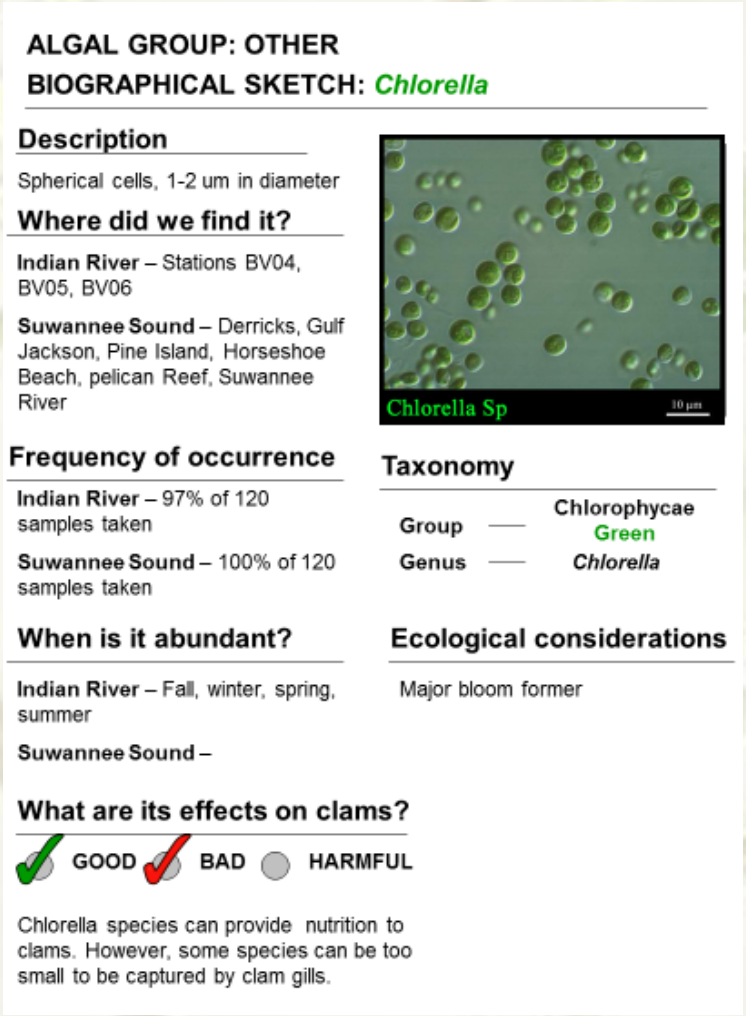
Hard clams are bivalve mollusks, animals that feed by filtering suspended particles out of the water column. Particulate material in shallow coastal waters is abundant, and the filtering capacity of bivalves often promotes good water quality. Filter feeding is accomplished by capturing food particles that enter the bivalve shell through water currents or active pumping by the animal. Particles are trapped by gill filaments that contain small hair-like protrusions called "cilia". The cilia then move the food to the mouth to be digested by the animal. If a particle is too small (<1 µm) it may pass through the filter, but if it is too big (>100-200 µm) it will be rejected as psuedofeces. Organic particles in the correct size range are either plankton, detritus (decaying plant material), or bacteria. Studies have shown that phytoplankton (microscopic algae) are the primary food source of most filter-feeding bivalves. Although most species of phytoplankton are acceptable food items, some produce toxins that can be harmful to hard clams and/or humans that consume them, and some species are inedible due to their physical structure. Phytoplankton can also vary in terms of chemical composition, such as lipid content, which can affect their quality as a food item.

What do Clams Eat? Online Guide



The "What Do Clams Eat" guide contains graphs depicting biomass patterns for the Indian River Lagoon and Suwannee Sound regions of Florida. Each graph illustrates the composition of four major groups of phytoplankton.

A list of example phytoplankton species is provided for each of the four major groups. When you click on a species in the list, you will find a biographical sketch with information about what the species looks like, where and how often we found it in our study, and the potential "good" and "bad" effects on clams. Most species have the potential to harm clams if they form dense blooms; however, the "good" and "bad" categories on this page refer to the acceptability of individual cells as food items.



SRAC
Southern Regional Aquaculture Center

SRAC Publication No. 5004
September 2010

Phytoplankton Culture for Aquaculture Feed

LeRoy Creswell[1]

Phytoplankton consists of one-celled marine and freshwater microalgae and other plant-like organisms. They are used in the production of pharmaceuticals, diet supplements, pigments, and biofuels, and also used as feeds in aquaculture. Phytoplankton are cultured to feed bivalve molluscs (all life stages), the early larval stages of crustaceans, and the zooplankton (e.g., rotifers, copepods) that are used as live food in fish hatcheries.

Flagellates and diatoms are two important types of phytoplankton at the base of the food chain. They manufacture cellular components through the process of photosynthesis, taking up carbon dioxide and nutrients from the water and using light as an energy source.

The microalgae used as feed in hatcheries vary in size, environmental requirements, growth rate, and nutritional value (Fig. 1, Tables 1 and 2) (Helm et al., 2004). When selecting a species for culture, it is important to take all of these parameters into consideration. Most hatcheries grow a variety of species that serve different needs throughout the production cycle with respect to size, digestibility, culture characteristics, and nutritional value (Muller-Feuga et al., 2003).

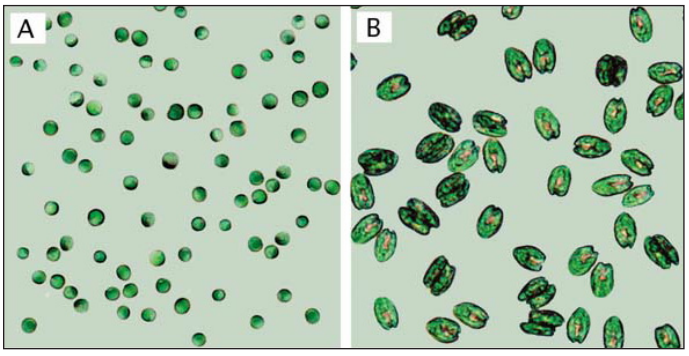


Figure 1. Photomicrographs of two popular species of microalgae commonly cultured in bivalve hatcheries. A) *Isochrysis* sp. (4–6 µm x 3–5 µm, and B) *Tetraselmis* sp. (14–20 µm x 8–12 µm) (Helm et al., 2004).

Table 1: Cell volume, organic weight, and gross lipid content of some commonly cultured phytoplankton species used in bivalve mollusc and fish hatcheries (Helm et al., 2004).

Species	Median cell volume (µm³)	Organic weight (pg)	Lipid content (%)
FLAGELLATES			
Tetraselmis suecica	300	200	6
*Dunaliella tertiolecta**	170	85	21
Isochrysis galbana (T-ISO)	40 – 50	19 – 24	20 - 24
DIATOMS			
Chaetoceros calcitrans	35	7	17
Chaetoceros gracilis	80	30	19
Thalassiosira pseudonana	45	22	24
Skeletonema costatum	85	29	12
*Phaeodactylum tricornutum**	40	23	12

* Species considered to be of poor nutritional value.

Table 2: Temperature, light, and salinity ranges for culturing selected microalgae species (Hoff and Snell, 2008).

Species	Temperature (°C)	Light (Lux)	Salinity (ppt – ‰)
Chaetoceros muelleri	25 – 30	8,000 – 10,000	20 - 35
Phaeodactylum tricornutum	18 – 22	3,000 – 5,000	25 – 32
Dicrateria sp.	25 – 32	3,000 – 10,000	15 – 30
Isochrysis galbana	25 – 30	2,500 – 10,000	10 – 30
Skeletonema costatum	10 – 27	2,500 – 5,000	15 – 30
Nannochloropsis oculata	20 – 30	2,500 – 8,000	0 - 36
Pavlova viridis	15 – 30	4,000 – 8,000	10 – 40
Tetraselmis tetrathele	5 – 33	5,000 – 10,000	6 – 53
Tetraselmis subcordiformis	20 – 28	5,000 – 10,000	20 - 40
Chlorella ellipsoidea	10 – 28	2,500 – 5,000	26 - 30

[1]University of Florida Sea Grant

Culture conditions can vary widely—from outdoor ponds or raceways with nutrients added to promote a bloom of the natural microalgae, to monocultures reared indoors under controlled environmental conditions. This paper focuses on the monoculture of microalgae under clearly defined environmental conditions and production protocols.

Microalgal culture facilities typically use seawater enriched with nutrients—primarily nitrates, phosphates, essential trace elements, vitamins, and, in the case of diatoms, silicates. Water used to culture microalgae should have similar chemical composition to that used to culture the animals, and it should be pretreated. Some laboratories use synthetic seawater for small-scale cultures, but it is prohibitively expensive for large-scale production in commercial hatcheries.

Population dynamics

Algal cells from a starter culture are inoculated into a larger volume of treated, enriched water to reach an initial low density of about 30 to 100 cells/µL. For the first 2 to 3 days the cells become acclimated to the new medium, grow, and begin cell division. This phase, termed the lag phase, varies in length depending on the amount of inoculum used (initial cell density), alga species (inherent division rate), irradiance, and temperature (Fig. 2). Once acclimated, the algal cells divide at an accelerating rate, and the population increases logarithmically; this *exponential growth phase* lasts 4 or more days. The cells are usually harvested for feeding during this phase. The *exponential growth phase* is followed by the *stationary phase,* when cell division declines and there is no further increase in cell density. This decreased growth is the result of changes in the concentration of nutrients, self-shading (high cell density reduces the amount of light available to algal cells), and changes in the culture medium, such as increasing pH and the build-up of metabolic waste products or substances called autoinhibitors that are secreted by some species (mostly diatoms). As the culture ages, the *stationary phase* is followed by a *senescent phase* in which the density of the culture will decline.

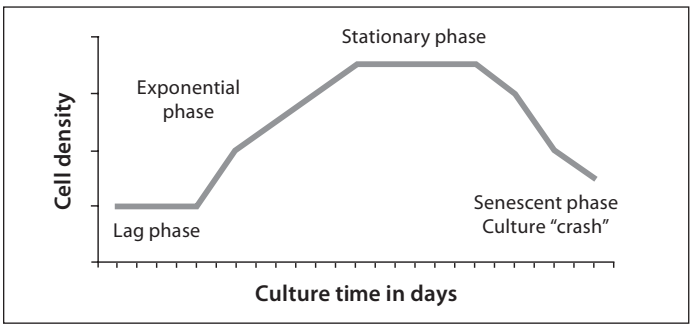


Figure 2. Phases in the growth of algal cultures.

Stationary phase algae should not be used for larviculture because although the algae may be nutritious, as they die the cells rupture and bacteria can proliferate (including some pathogenic bacteria such as *Vibrio* spp.). The wise culturist knows that the line between feeding larvae and poisoning them can become blurry as algal cultures age.

The culture environment

When designing a microalgal production system, consider which species is most appropriate for the intended use (e.g., size and nutritional characteristics). Also consider yield, operating costs, and reliability. Microalgal culture is the most expensive and technically challenging aspect of all hatchery operations. The cost of producing microalgal feed ranges from $100 to $400 per dry kilogram ($45 to $180 per pound) of microalgal biomass (Wikfors, 2000). Algal culture accounts for about 40 percent of the cost of rearing bivalve seed to a shell length of 5 mm in a land-based hatchery (Ukeles, 1980).

Hatcheries use either intensive indoor culture with artificial lighting or extensive outdoor culture in large tanks, raceways, or ponds with natural lighting. Some hatcheries use a combination of the two. Intensive indoor systems are expensive and labor intensive, but they are more reliable and more productive (relative to space requirements) than outdoor systems. Open ponds and raceways are also more prone to biological contamination or other water quality problems. As one might imagine, the potential for "culture crashes" increases as the degree of control over environmental factors such as temperature, illumination, nutrient availability, pH, and potential contamination decreases.

The nutritional value of algae is affected by culture age and growth phase, light characteristics and intensity, nutrient limitation and source, and cell density (Depauw and Persoone, 1988). Whether intensive or extensive, microalgal culture requires filtered and treated water, nutrients, a light source, aeration and mixing, temperature/salinity control, pH control, and a high-quality inoculum to ensure a satisfactory yield (Fig. 3).

Filtered and treated water

Pretreatment of water, whether saltwater or freshwater, is one of the most important steps in successful microalgal culture. Culture water should be free of suspended solids, plankton (e.g., protozoans, ciliates and other algae species), bacteria, unacceptably high concentrations of dissolved organic compounds (DOC), dissolved metals, and pesticides.

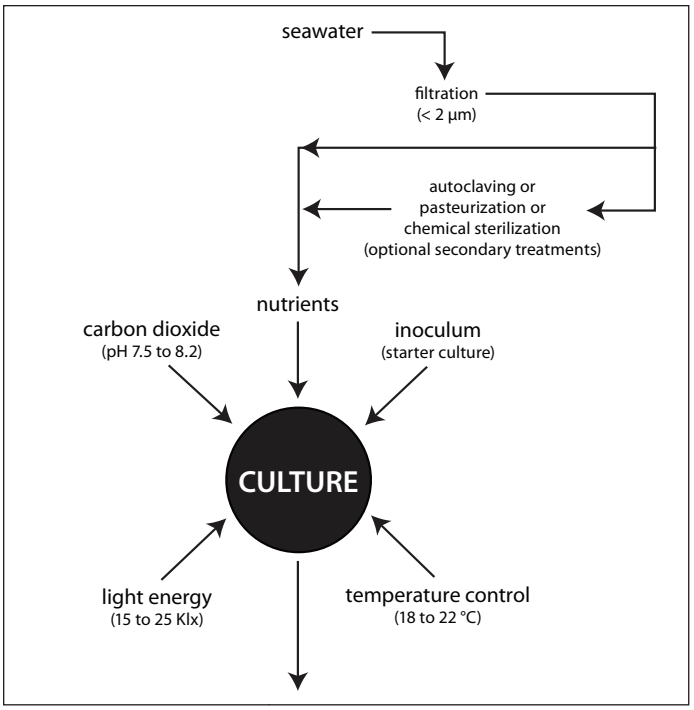


Figure 3. Input requirements and environmental criteria for microalgal cultures. Optimal temperature, salinity, and light intensity may vary with species (e.g., tropical vs. temperate microalgae). Although tropical species grow more rapidly at warmer temperatures (25 to 30 °C), stock cultures are kept cooler to inhibit bacterial growth (Helm et al., 2004).

Pretreatment typically includes mechanical and chemical filtration, sterilization or disinfection, and nutrient enrichment. The choice of treatment should be based upon species cultured, volume requirements, and cost.

Mechanical filtration. Mechanical filtration removes suspended solids, plankton and bacteria and is usually used with the other forms of treatment described below. The type of mechanical filtration used depends on the condition of the incoming water and the volume of water to be treated. A mechanical filter usually consists of a series of filters that remove increasingly smaller particles—sand filters or polyester filter bags (20- to 35-µm), followed by cartridge filters (10-, 5-, 1-µm) or diatomaceous earth (DE) filters. Small volumes of seawater can be filtered to remove bacteria using 0.22- or 0.45-µm membrane cartridge filters.

Chemical filtration. Dissolved inorganic and organic compounds (DOC), metals, pesticides, and other contaminants can prevent or retard microalgal growth, although detecting them can be complicated and costly. Activated carbon (charcoal) filtration is helpful in reducing DOC, while deionization resins are effective in removing metals and hydrocarbons.

Heat sterilization. Pre-filtered seawater can be sterilized by autoclaving at 1.06 kg/cm^2 for 20 minutes. Autoclaving is most suitable for small volumes, while batch or continuous pasteurization at 65 to 70 °C is used for large volumes. Pasteurization at 50 °C for 8 to 10 hours is also effective; a glass-lined water heater or 500- to 1,000-W submersion heater can be used. Microwave sterilization is useful for small volumes of pre-filtered seawater (1 to 5 µm for 8 to 10 minutes per 1 to 1.5 L using a 700-W unit). Nutrients can be added before microwaving since the temperature will not exceed 84 °C (181 °F) (Hoff, 1996). Bellows and Guillard showed that using a 1.2-ft^3, 700-W microwave on high power would effectively kill microalgae in 5 minutes, bacteria in 8 minutes, and fungi in 10 minutes in a volume of 1.5 L of filtered (and unfiltered) seawater (Table 3).

Chemical sterilization. Chlorination is the simplest and most common method of chemical sterilization for culture volumes of at least 4 L. Pre-filtered seawater can be sterilized with sodium hypochlorite solution at 2.5 mg/L free chlorine by adding 1 to 5 mL of household bleach (5% sodium hypochlorite) per liter of seawater. Granular swimming pool chlorine is also effective; a dosage of 1 ounce (28 g) to 500 gallons (1,875 L) yields a similar concentration of chlorine as liquid bleach. Sterilization occurs in a short period of time, usually 10 to 30 minutes, although many culturists suggest a longer time (12 hours or overnight) for a margin of safety. Before use, neutralize the residual chlorine by adding an excess of sodium thiosulphate solution ($Na_2S_2O_3 \cdot 5H_2O$). If 250 g of sodium thiosulfate is dissolved in 1 liter of water, then 1 mL of the sodium thiosulfate solution added for every 4 mL of bleach used is sufficient to eliminate residual

Table 3: Summary of heat sterilization types, effective methods, application, and limitations (Kawachi and Noël, 2005)

Sterilization method	Effective method	Application	Limitation
Autoclaving	121°C at 29.4 psi (2 atm) @ 10 minutes for test tubes @ 1 hour for 10 liters liquid	Liquids and agar, glass and metal vessels	Avoid non-heat-resistant materials; pH change, metal contamination
Pasteurization	65–80 °C followed by quick cooling (4–10 ° cooling) 50 °C for 8–10 hours using glass-lined water heater, 500- to 1000-W immersion heater	Liquids with heat labile components	Not complete sterilization
Microwave	10 minutes/L of liquid with 700-W microwave; 20 minutes at 600-W, 45 minutes for dry goods	Small volume liquids (including media), dry goods, and vessels	Limited volume capability

chlorine (dechlorination). Common swimming pool chlorine test kits can be used to determine the presence of residual chlorine, but they do not give a precise measure of chlorine concentration; as a general practice, additional sodium thiosulfate solution should be added if there is any indication of residual chlorine.

Ultraviolet irradiation (UV) and ozone (O_3) disinfection. Either UV or ozone can be used to disinfect culture water, although both are most effective after mechanical filtration has removed suspended particulates. It should be noted that "sterilization" is defined as the absolute destruction of all microbial organisms (including bacterial spores), while "disinfection" does not eliminate all microbes but reduces their numbers to a level where the risk of infection is small enough to be acceptable.

UV is the more common of the two, largely because it does not leave concentrations of hazardous by-products. Ozone at high levels can produce chloramines, which are toxic to marine animals. Ozone released into the air can be a safety hazard (if you can smell a faint chlorine smell, residual ozone is present and may be hazardous to your health).

Ozone is a strong oxidizing agent that is particularly effective in removing dissolved organics, pesticides, color and nitrates. It is highly unstable and quickly reverts to O_2, but it is also highly corrosive and must be handled with special materials. In-line ozone generators are the most common and usually have monitors/controls to provide an adequate level of ozone yet avoid residual build-up. However, because there is a risk of introducing ozonated water into the culture system, as well as safety concerns for hatchery staff, ozone is not recommended for operators who lack experience and the monitoring equipment to properly manage ozone levels.

Ultraviolet radiation (germicidal energy) is an efficient, simple and reliable way to kill microorganisms in culture water. With proper exposure in clear water, ultraviolet light kills a microorganism by penetrating its cell wall and destroying its nuclear material. Low-pressure mercury vapor UV bulbs are best suited for disinfection because their spectral wavelength (254 nm) is close to the most efficient germicidal wavelength (265 nm). However, the killing power of UV is affected by turbidity/coloration of the incoming water, distance from the source, exposure time (flow rate), species, and age of the bulb (some lights age rapidly, losing as much as 40 percent of their wattage after 6 months). Minimum dosages vary widely for different microorganisms: 15,000 µwatt-sec/cm^2 for most bacteria, 22,000 µwatt-sec/cm^2 for water-borne algae, 35,000 µwatt-sec/cm^2 for bacteria/viruses, 100,000 µwatt-sec/cm^2 for protozoans, and as much as 330,000 µwatt-sec/cm^2 for *Aspergillus niger* (mold) (Depauw and Persoone, 1988).

Wattage and flow rate are the most important factors in achieving UV sterilization; the slower the flow rate, the higher the kill rate for a given bulb wattage (Escobal, 1993). For example, a 40-watt mercury vapor bulb with a flow rate of 500 gallons per hour in a 2-inch-diameter pipe will deliver approximately 11,530 µwatt-sec/cm^2. Increasing the pipe diameter to 3 inches (thereby reducing flow rate) will increase the dosage to 17,530 µwatt-sec/cm^2; cutting the flow rate in half in that 3-inch pipe further increases the dosage to 34,340 µwatt-sec/cm^2 (Hoff et al., 2008).

Nutrient enrichment

The objective of culturing microalgae is to obtain the highest cell densities in the shortest period of time, and natural concentrations of nutrients in freshwater and seawater are usually insufficient to support high algal yields. Although trace elements are usually found in sufficient quantities, macronutrients are in short supply (usually phosphorus in freshwater and nitrate in saltwater). Several nutrient enrichment media containing soil extract, nitrates, phosphorus, trace elements, and vitamins have been described for freshwater and saltwater (Creswell, 1993). Of the nutrient media formulations used to culture marine microalgae in laboratories and hatcheries, Guillard and Ryther's F/2 media is the most widely used, and a pre-mixed solu-

Table 4: Guillard's F/2 media used to culture marine microalgae (Guillard, 1975).

Major nutrient	**Chemical formula**	**Concentration (gram/liter)**
# 1. Nitrate	$NaNO_3$	75.0 g/L
# 2. Phosphate	$NaH_2PO_4 \cdot H_2O$	5.0 g/L
# 3. Silicate	$Na_2SiO_3 \cdot 9H_2O$	30.0 g/L
# 4. **Trace Metals**		
	$FeCl_3 \cdot 6H_2O$	3.5 g
	Na_2EDTA	4.36 g
Dissolve in 900 ml of distilled H_2O. Add 1 ml of each of the following trace metal solutions.		
	$CuSO_4 \cdot 5H_2O$	0.98 g/100 ml
	$ZnSO_4 \cdot 7H_2O$	2.20 g/100 ml
	$CoCl_2 \cdot 6H_2O$	1.00 g/100 ml
	$MnCl_2 \cdot 4H_2O$	18.00 g/100 ml
	$Na_2MoO_4 \cdot 2H_2O$	0.63 g/100 ml
Make up the volume to 1 liter with distilled H_2O. Add 1 ml per liter of seawater of the above solutions # 1–4.		
# 5. **Vitamins**		
	Biotin	1.0 mg
	B_{12}	1.0 mg
	Thiamin HCl	20.0 mg
Dissolve vitamins in 1 liter of distilled H_2O. **Store frozen.** Add 0.5 ml of vitamin solution for every 1 liter of seawater.		

tion is available from a variety of vendors (Table 4). There are dozens of culture media recipes, many of which were formulated specifically for certain types/species of microalgae and cyanobacteria. A good reference is *Algal Culturing Techniques,* edited by R. A. Anderson. Table 5 lists services that have culture medium recipes on their websites.

Table 5: Major service culture collections with culture medium recipes on their websites (Anderson, 2005).

Culture collection	Website URL
CCAP	*www.ife.ac.uk/ccap*
CCMP	*ccm.bigelow.org*
NIES	*www.nies.go.jp/biology/mcc/home.htm*
PCC	*www.pasteur.fr/recherche/banques/PCC*
SAG	*www.epsag.uni-goettingen.de/html/sag.html*
UTCC	*www.botany.utoronto.ca/utcc*
UTEX	*www.bio.utexas.edu/research/utex*
For additional culture collections	*http://wdcm.nig.ac.jp/hpcc.html*

Light source

Light is the energy source that drives photosynthesis to convert nutrients into algal biomass. Maximum culture depth and cell density are the primary variables regulating the efficient use of light (Richmond et al., 1980). Light intensity, spectral characteristics, and photoperiod are the components of an illumination regime. Indoor microalgal facilities usually use fluorescent "cool white" bulbs (2,500 lux), while outdoor systems and greenhouses use ambient sunlight in combination with fluorescent or metal halide bulbs to provide evening illumination. The spectral characteristics of "cool white" bulbs are not ideal for intensive microalgae production; bulbs with enhanced red and blue wavelengths (Gro-Lux™) support higher yields. The age of the bulb is also important, as the spectral characteristics and luminosity change over time; bulbs should be replaced at least annually.

Irradiation of 2,500 to 5,000 lux (250 to 500 footcandles) is optimal for microalgae photosynthesis, with a maximum of 10,000 lux (Escobal, 1993). Guillard (1975) recommended 3,500 and 4,500 lux for stock culture of *Thalassiosira pseudonana* under continuous and 14 hours per day illumination, respectively. In indoor facilities, bulbs should be 6 to 10 inches from stock cultures; if possible, the ballasts should be outside the culture room to help maintain temperature control.

Internally illuminated culture vessels are costly to construct but inexpensive to operate. Mounting the lamps inside a glass or clear plastic cylinder within the culture vessel reduces the distance the light must travel to penetrate the culture. Culture cylinders with internally mounted lights typically produce as much as cultures with three times the volume.

Metal halide lamps (750 to 1,000 W) are usually used to illuminate larger cultures (1,800 liters or more), and since they generate considerable heat, they should be placed at least 12 inches above the surface in open, well-ventilated greenhouses. If natural light is being used for large-volume cultures in greenhouses, it is best to use the morning sun, with 40 percent shade cloth on the west side of the building and 60 percent shade cloth down the middle of a north-south oriented building, especially in summer.

Although most commercial light meters measure "lux," many references in the literature related to light requirements for phytoplankton culture prefer to express optimum irradiance in terms of "Photosynthetically Active Radiation" (PAR), which is expressed as µmol photons $\cdot s^{-1} \cdot m^{-2}$, radiation within wavelengths of 400 to 700 nm. Converting lux values to PAR depends on the type of lamp and its spectral characteristics. Multiply lux by the following conversions for PAR:

- incandescent = 0.019
- metal halide = 0.014
- cool white flourescent = 0.013
- daylight flourescent = 0.014
- GRO flourescent = 0.029
- clear day sunlight = 0.018

(source: *http://www.egc.com*)

Artificial light is usually preferred over sunlight. With sunlight, the duration and intensity are not easily controlled, which may cause overheating, insufficient irradiance, or photoinhibition if the light is too intense for too long (Escobal, 1993). Artificial lighting can be controlled with a simple timer or light monitor and should be set for a minimum of 16:8 hours light/dark per day (minimum) to 24-hour illumination (maximum) for indoor cultures. Although artificial lighting can be precisely controlled in terms of quality and quantity, it is costly and accounts for almost 95 percent of the cost to culture microalgae (Muller-Feuga et al., 2003).

Temperature control

Because most of the microalgae species preferred by culturists are tropical/subtropical, most strains grow best at temperatures ranging from 16 to 27 °C (60 to 80 °F). The optimum is about 24 °C (75 °F). Ukeles (1976) compared the growth response of several microalgae species to temperature (Table 6). The optimum temperature for growth will vary with species, and to some extent is a complex factor that depends on other environmental conditions. Cultures should be maintained at the lowest temperature that is consistent with good yield to avoid encouraging bacterial growth. When considering tem-

Table 6: Growth response of different microalgal species to various temperatures (°C). Growth rates are relative to performance of a control cultured at 20.5 °C (Ukeles, 1976).

Species	No growth	Growth less than control	Growth equal to control at 20.5 °C	Growth less than control	No growth
Monochrysis lutheri	8–9	12	14–25	27	29–5
Isochrysis galbana	8–9	12	14–22	24–25	27–35
Phaeodactylum tricornutum	–	–	8–24	27	29–35
Dunaliella euchlora	8–9	–	12–35	–	39
Platymonus (Tetraselmis sp.*)*	–	8–9	12–32	–	35
Chlorella sp. (isolate # 580)	8–9	12	14–35	–	–
Chlorella sp. (UHMC isolate)	8–9	12	14–29	–	32–35

perature characteristics for an enclosed culture room, one should consider: 1) the size of the room, 2) heat sources (such as lights and ballasts), and 3) the volume and temperature of air pumped into the culture vessels.

Aeration and mixing

Aeration is important for microalgal culture because: 1) air is a source of carbon (from CO_2) for photosynthesis; 2) CO_2 provides essential pH stabilization; and 3) physically mixing the culture keeps nutrients and cells evenly distributed, reduces self-shading and/or photoinhibition (a decrease in photosynthesis due to excess light), and avoids thermal stratification in outdoor systems. Air diffusers (airstones) create small bubbles that maximize oxygen/CO_2 transfer, and they are frequently used for small-volume cultures. In larger culture containers, fine bubbles from air diffusers create spray and foam that can promote bacterial growth; larger bubbles (no airstones) actually do a better job of mixing the culture with minimal foaming. Common alternatives for mixing larger volume cultures include jet pumps, paddle wheels, continuous recirculation, and air-lift pumps (Persoone et al., 1980).

Carbon dioxide (CO_2) source and pH control

Carbon dioxide plays a dual role in microalgal culture. It provides a source of carbon to support photosynthesis, and it helps maintain pH at optimum levels (7.5 to 8.2 for marine species). As culture density increases, more carbon is consumed through photosynthesis, reducing CO_2 concentration and causing the pH to rise. At about pH 10 some nutrients will precipitate, algal growth will be retarded, and the culture could completely collapse. This can be prevented if the pH is maintained by introducing CO_2 into the air delivery system. This can be done manually (while the cultures are illuminated), pulsed intermittently using a timer and solenoid valve, or, most effectively, by using a pH monitor/controller.

Inoculum

Most hatcheries will culture several species of microalgae to provide live feeds with different sizes and nutritional characteristics, depending on the animal being cultured and its life stage. The culture protocol for each species will be dictated by the characteristics of the microalgae (e.g., growth rate and environmental requirements), harvest yields, and use requirements.

Maintaining and transferring stock and starter cultures

Stocks of monospecific (uni-algal) cultures can be obtained by collecting local species, separating them by size (filtration) or density (centrifugation), and inoculating agar plates containing enrichment media. From these multi-species algal cultures, individual colonies are selected through agar streaks, micropipette isolation, liquid dilution, or flow cytometer cell sorters (Fulks and Main, 1991). Culturing algae in highly filtered, autoclaved, enriched seawater in the presence of antibiotics allows bacteria- and protozoan-free *pure cultures* (axenic) to be isolated. However, the isolation and screening of local species is laborious and mono-specific cultures of most microalgae species used for aquaculture are readily available from research laboratories, commercial hatcheries, and vendors.

Stock cultures are kept in specialized maintenance media, which may be enriched seawater or nutrient-enriched agar plates or slants, under closely controlled conditions of temperature and illumination. A special temperature-controlled area or room adjacent to the algal culture room is usually allocated for this purpose.

Stock cultures serve as inocula for the large-volume production of phytoplankton used for harvest or feeding. Stock cultures containing sterile, autoclaved media are kept in small, transparent, autoclavable containers such as 25-mL test tubes or 250- to 500-mL borosilicate glass, flat-bottomed, conical flasks fitted with cotton wool plugs at the necks (or polyethylene beakers can serve as caps). They also are maintained in seawater agar medium impregnated with suitable nutrients in petri dishes or on slants in test tubes. Every effort should be made not to contaminate the stock and starter cultures with competing microorganisms. To minimize potential contamination, an enclosed culture transfer hood outfitted with a Bunsen burner and UV lights should be used (a laminar-flow hood is preferred if available) (Fig. 4).

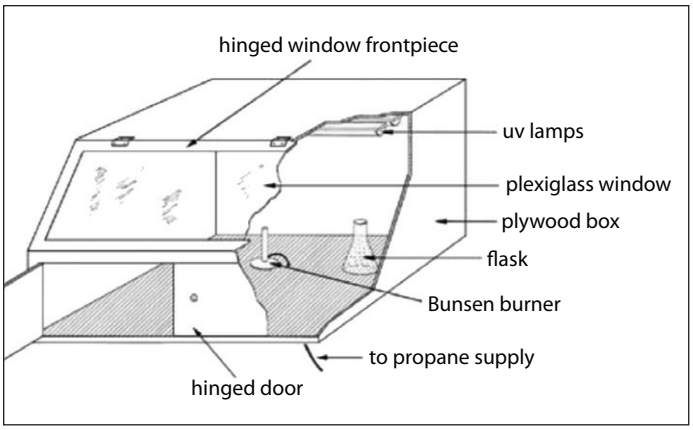


Figure 4. A schematic diagram of a stock culture transfer chamber (Helm et al., 2004).

The sterile procedures described below should be followed.

1) Wipe all inner surfaces of the inoculating hood and working surfaces with 70 percent ethanol.
2) Place all flasks that will be used in the hood, including flasks to be transferred from (the transfer flask) and flasks containing sterilized media that will be inoculated under the culture transfer hood.
3) Irradiate flasks to be inoculated with an ultraviolet lamp for at least 20 minutes. Be sure the hood has a dark cover over the viewing glass (UV radiation can be damaging to the eyes).
4) Switch off the UV lamp; ignite a small Bunsen burner; remove caps from one transfer and one new flask; and flame the neck of each flask by slowly rotating the neck through the flame.
5) Tilt the neck of the transfer flask toward the new flask. In one motion, remove both stoppers and pour an inoculum into the new flask. Transfer approximately 50 mL for diatom species and 100 mL for flagellates. Avoid touching the necks of the two flasks. Never touch the portion of the stopper that is inserted into the flask. Once the inoculum is added, replace the stopper in the transfer flask. Slowly flame the neck of the new flask before replacing its stopper.
6) Replace the cap over the neck of the new flask and use a waterproof marker pen to label the new flask with the algal species inoculated and the date of transfer.
7) After all inoculations are completed, turn off the burner and transfer all new flasks to an algal incubator or a well-lit area in the algal culture facility. The remaining inoculum in the transfer flasks can be used to inoculate larger cultures such as 4-L flasks or carboys.
8) Empty test tubes, flasks, stoppers and/or caps should be removed, thoroughly washed, and sterilized or discarded.
9) Remove all materials from the working area and wipe the surface with 70 percent ethanol.

If you are transferring liquid cultures using glass Pasteur pipettes, follow these steps (Kawachi and Noel, 2005):

1) Bring the pipette canister (used to sterilize the pipettes) close to the Bunsen burner, the cap removed, and gently shake it so that one pipette is extruded a few centimeters from the canister opening.
2) Remove the pipette from the canister carefully so that its tip does not come in contact with the canister opening.
3) Replace the canister lid. Place the pipette bulb adjacent to the Bunsen burner and clean the inside with 70 percent ethanol.
4) Place the bulb on the pipette, pick up the cell culture vessel, and flame at an angle of at least 45 degrees.
5) Remove the vessel from the flame, insert the tip of the pipette into the liquid, being careful not to touch the sides of the vessel, and collect the desired amount of inoculum by controlling the pressure of the bulb.
6) Remove the pipette, orienting it in an almost horizontal position; reflame the mouth of the vessel, and replace the cap or plug.
7) Using the same procedure, open the new vessel, flame the opening, and insert the pipette into the new vessel without touching the mouth.
8) Slowly discharge the cell suspension, remove the pipette, flame the mouth of the vessel, and replace the cap.
9) Remove the pipette bulb and place the used pipette into a discard container to be discarded or reused. Clean the bulb with 70 percent ethanol for reuse.
10) Once all transfers are completed, turn off the Bunsen burner, remove all materials from the working area, and wipe the surface with 70 percent ethanol.

Progressive batch culture

The quantities of algae cells required for feeding mollusc larvae and other zooplankton are produced through a process called *progressive batch culture* (transferring small-volume cultures of concentrated inoculum into larger volumes of treated, enriched water). Starting with cells taken from an axenic stock culture (test tubes), microalgae are cultured in an enriched medium through a series of culture vessels of increasing volume (Fig. 5). The

algae grown in each culture vessel serves as the inoculum for the next larger vessel, until the quantity of cells required for feeding is reached. This is a typical series for large-scale production:

1) 25-mL test tubes (10-mL stock culture) inoculates….
2) 500-mL flasks (250-mL starter culture) inoculates….
3) 2.8- to 4-liter flasks (1,000-mL culture) inoculates….
4) 20-liter carboys (16-liter culture) inoculates….
5) 250-liter cylinders (180-liter culture) inoculates….
6) 12,000-liter tanks (10,000-liter culture) inoculates….

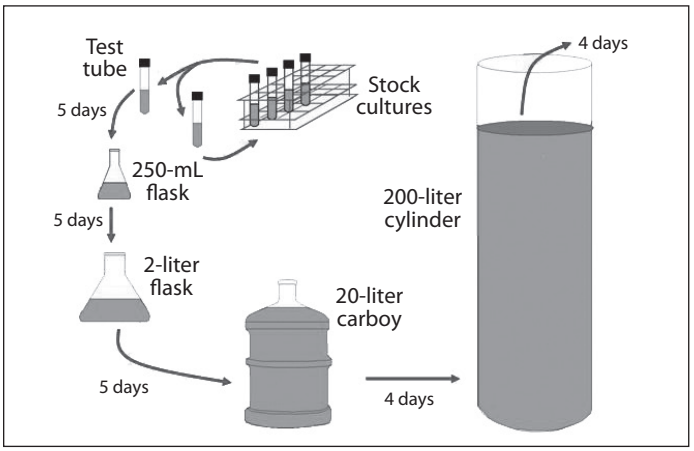


Figure 5. Serial dilution of microalgae from stock culture (test tubes) to 200-L fiberglass cylinders with the approximate duration between each transfer (courtesy P. van Wyk, Harbor Branch Oceanographic Institute).

Starter cultures are used to inoculate "intermediate cultures" (2 to 25 L), which are used to inoculate even larger volume cultures for final production before harvest and feeding. Similar to stock cultures, starter cultures can be grown in 500-mL flasks with 250 mL of sterile medium; about 50 mL of the starter culture is transferred to similar volume flasks to maintain the line, while the remaining 200 mL are used to inoculate intermediate culture containers (typically from 4-L flasks to 20-L carboys) (Fig. 6).

Procedures for maintaining starter cultures are almost identical to those described above for stock cultures. A line of starter cultures is originally established from the stock culture of the required species. They are grown at 18 to 22 °C at a distance of 15 to 20 cm from 65- or 80-W fluorescent lamps, giving a level of illumination at the culture surface of 4,750 to 5,250 1ux. Small-volume cultures (test tubes to 1-L flasks) are usually manually shaken daily to facilitate gas exchange and mixing. Aeration is required

Figure 6. Starter cultures in 500-ml flasks and 2-L and 4-L polycarbonate containers. Note that the flasks are not aerated, but the larger containers are aerated (with CO_2 added) and contain an in-line air filter (J. Scarpa, Harbor Branch Oceanographic Institute).

for 2-L flasks and larger volumes, and in-line filters on the delivery tubing are necessary to prevent contaminants that can be introduced through aeration. Starter cultures are generally aerated with an air/CO_2 mixture to maintain a satisfactory pH and provide addi-tional carbon for photosynthesis. When CO_2 is used, the pH is usually maintained between 7.5 and 8.5.

Stock cultures are grown for varying periods before inoculation in 500-mL flasks. For diatom species this period is 3 to 5 days; for the majority of flagellates it is 7 to 14 days. When ready for use, 20 to 50 mL of the starter culture (depending on species and cell density) is transferred to a fresh 250-mL culture to maintain the starter culture line. The remainder is used as inoculum for larger cultures (usually 1,000 mL in 2.8- to 4-L flasks) to be grown for feeding or as an intermediate step in large-scale culture, where they are used as inocula for 20- to 40-L cultures. To maintain high-quality cultures, transfers should be made during the exponential growth phase, with an inoculum of at least 10 to 20 percent of the total volume or an initial concentration of about 105 cells/mL, to promote rapid population growth.

Throughout the scale-up process, contamination is a constant threat and cleanliness and attention to detail are

critical. Contaminants may be chemical or biological and they can originate from one or several sources. A common chemical contaminant is residual chlorine from the sterilization process, while biological contaminants might include: 1) excessive levels of bacteria (indicated by cloudy water), 2) protozoans or rotifers (culture water turns off color and clears), 3) competing microalgae (color change or crust attached to culture vessel walls), and 4) macroalgae (green or brown strands attached to culture vessel walls). Identifying bacterial and microalgal contaminants usually requires 100X to 400X magnification, while protozoan contamination can be observed under 15X to 40X magnification (Hoff and Snell, 2008). Possible sources of contamination are shown in Figure 7.

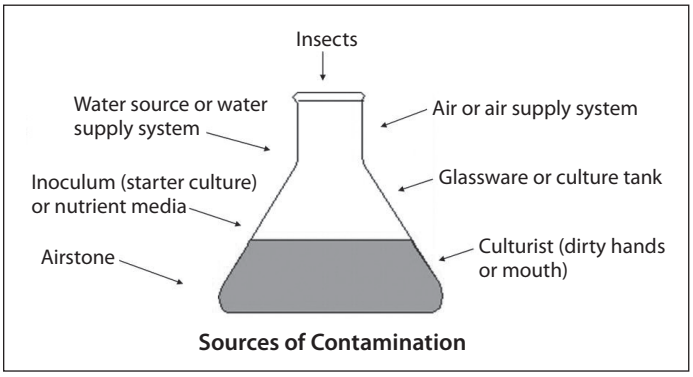


Figure 7. Possible sources of contamination (adapted from Hoff and Snell, 2008).

Estimating algal density

Estimating algal density is an inherent part of any algal production system. Algal biomass is the criterion used to determine when to transfer inoculum through serial dilution to larger volume cultures and to determine harvest volumes of cultures in production. For stock and starter cultures, the most accurate measurement of cell density can be made using a Palmer-Maloney slide or a hemacytometer.

The chamber of the Palmer-Maloney slide is without rulings and is circular. It is 17.9 mm in diameter, 400 µm deep, and has an area of 250 mm^2, for a total volume of 0.1 mL. Even very small microalgae at low concentrations (10 per mL) can be detected. Hemacytometers are thick glass slides with two chambers on the upper surface, each measuring 1.0 x 1.0 mm. A special coverslip is placed over these two chambers, giving a depth of 0.1 mm and a total volume in each chamber of 0.1 mm^3. The base of each chamber is marked with a grid to aid in counting cells within the area (Fig. 8). Before counting motile algal species, one or two drops of 10 percent formalin should be added to a 10- to 20-mL sample of the culture to be

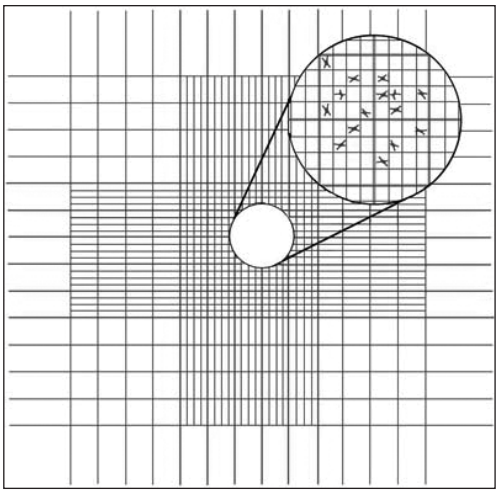


Figure 8. A hemacytometer used to count microalgae cells (Helm et al., 2004).

counted. With the coverslip in position, one or two drops of the algal sample are introduced by means of a Pasteur pipette to fill both chambers.

The central grid of each chamber is subdivided into 25 squares, each measuring 0.2 x 0.2 mm; these are further subdivided into 16 squares (0.05 x 0.05 mm). Therefore, the volume of each grid is 0.2 x 0.2 x 0.1 mm = 0.004 mm^3. To determine cell density:

- Count the number of cells in ten randomly selected 0.2 x 0.2 mm grids and calculate the average (as an example, an average of 42.5 cells/grid).
- Multiply the average (42.5 cells) by 250 to get 10,625 cells/mm^3 (0.004 mm^3 x 250 = 1 mm^3).
- Since there are 1,000 mm^3 in 1 mL, multiply the value in the second step by 1,000 to get cells/mL. 42.5 x 250 x 1,000 = 10,625,000 cells/mL, 10.625 million cells/mL (10.62 x 10^6).

The Coulter Counter, now called a "multisizer," was originally developed to count blood cells. Algal cells pass through a small aperture (2 to 10 µm) and a slight electrical current travelling between two electrodes. Each time a cell passes between the electrodes, the current is impeded and the cell is counted. The advantages of the Coulter Counter are its accuracy and efficiency; the disadvantages are that it does not discriminate between algal cells and other particles, dense culture needs to be diluted to get an accurate count, and they are expensive.

For larger cultures, a spectrophotometer or fluorometer that measures the chlorophyll α content in an algal culture can be used to obtain a quick approximation of cell density. Graphs comparing cell density and readings on either instrument must be prepared for each algal species. However, the chlorophyll α content in an algal cell is not constant and varies with the nutritional state of the

cell. This will affect the accuracy of cell density estimates derived with these instruments.

An inexpensive way to estimate algal density in large cultures is to use a "Secchi disk," a technique that has been used by field biologists for decades. Once calibrated to the microalga species in culture, Secchi disks can provide a reasonably accurate estimate of algal cell density (Hoff and Snell, 2008).

Intermediate culture

Intermediate culture volumes, typically 4-L flasks to 20-L carboys, are used to inoculate larger vessels, typically 100- to 200-L translucent fiberglass cylinders or polyethylene bags, or even larger fiberglass tanks and raceways. The complexity of the culture operation depends on the requirement for algae and cost constraints. The simplest culture system may be just a scaled-up version of the starter cultures using 4-L flasks or 20-L carboys. Sterile, nutrient-enriched seawater with an inoculum should be aerated with a mixture of 2 percent CO_2 carried in compressed air. Illumination for culture growth is provided by fluorescent lamps, usually mounted externally to the culture flasks. The number of lamps used is determined by the height and diameter of the culture vessels, with the object of providing 15,000 to 25,000 lux measured at the center of the empty culture container. Two 65- or 80-W lamps are sufficient to illuminate 3-L glass flasks, which are about 18 cm in diameter, whereas five lamps of the same light output are necessary for 20-L carboys (Fig. 9).

Cultures 4 to 8 days old from carboys (20-L) are used to inoculate 200-L translucent fiberglass cylinders or polyethylene culture bags. In most cases, these larger volume cultures are housed in greenhouses and receive natural light (adequate illumination from fluorescent bulbs is usually cost prohibitive). The vessels are filled with filtered, UV-irradiated, and usually chlorinated/dechlorinated seawater, enriched and inoculated. Each cylinder is carefully labeled to document the date of the sterilization process, the enrichment, and the species inoculated. Under optimal environmental conditions, the culture will be harvested in 4 days or used to inoculate large-scale cultures in tanks or outdoor ponds. (Fig. 10).

Figure 9. Carboys (20-L) are "intermediate" cultures that will be used to inoculate 200-L fiberglass cylinders or fed directly to bivalve larviculture tanks (courtesy J. Scarpa, Harbor Branch Oceanographic Institute).

Figure 10. Translucent fiberglass cylinders (200-L) are commonly used for production of microalgae up to harvest and feeding. For large-scale algae production, they can be used to inoculate 2,000- to 5,000-L mass culture tanks.

Batch, semi-continuous, and continuous culture systems

Because microalgal culture produces high concentrations of cells, most laboratories require only small volumes of algae for food. These can be cultured in 4-L containers or 20-L carboys using "batch" culture protocols. Commercial hatcheries, which require much larger volumes of algae, often use semi-continuous or continuous culture systems.

Batch cultures are inoculated with the desired species that will grow rapidly under optimal conditions until the rate of cell division begins to decline, indicating the transition from the exponential phase to the stationary phase. At that point, the culture is completely harvested and the container is washed, refilled (with sterilized, enriched medium), and inoculated to begin a new culture. Batch culture is generally used for delicate species or for rapidly growing diatoms. Although batch culture is considered the least efficient method of production, it is predictable, and contamination is less likely than in semi-continuous cultures

that stay in production through several harvests. Because the entire culture is harvested, yield per tank is less than in semi-continuous systems; therefore, more tanks are required for the same level of production (Fulks and Main, 1991).

Semi-continuous cultures begin much the same way as the batch cultures, but instead of harvesting the entire volume, 25 to 50 percent of the volume is harvested at the point when light has become a limiting factor (late exponential phase). The harvested volume is then replaced with freshly prepared culture medium and the remaining algal cells serve as inoculum. Semi-continuous cultures grow rapidly and can be harvested every 2 or 3 days. In this way, the life of a culture can be extended; cultures of some hardier species, such as *Tetraselmis suecica,* will last for 3 months or more with harvests of 25 to 50 percent of the culture volume three times each week. Semi-continuous culture is mainly used with hardier species of flagellates. Semi-continuous cultures may be grown indoors or outdoors. Their longevity is unpredictable, especially outdoors, because competitors, predators, bacteria and/or other contaminants and metabolites build up and render the culture unsuitable (Guillard and Morton, 2003).

Droop (1975) defines continuous culture as "steady-state continuous flow cultures in which the rate of growth is governed by the rate of supply of the limiting nutrient." Continuous culture systems are delicately balanced so that culture organisms are harvested continually and the nutrient-enriched media is replenished continually, consistent with the growth rate (sustainable yield) of the culture.

In order to harvest algae continuously at a level adjusted to the maximum specific growth rate (exponential phase) of the culture species, two monitoring and control devices can be used—chemostats and turbidostats. In both cases, fresh, sterile media enters the culture vessel, displacing old media and algal cells that are harvested through an overflow port. Chemostats act on the principle of limited nutrients, so if the concentration of the limiting nutrient (e.g., nitrate) falls below a certain level, a fixed quantity of nutrient solution is added; algal growth rate is regulated by the limiting nutrient, not cell density, and flow is continuous (James et al., 1988). Turbidostats have photoelectric monitors connected to solenoid valves that control the withdrawal of algal suspension and the addition of fresh medium as a function of cell density (by measuring turbidity); the flow is not continuous. A variety of mathematical models have been developed that, theoretically, can maximize production from continuously harvested systems (based on algae growth rate, optimum dilution rate, and nutrient concentration), but in practice the culture manipulations are determined empirically after a series of several trials (Sorgeloos et al., 1976; Laing and Jones, 1988; Landau, 1992).

Culture in polyethylene bags

Heavy-gauge polyethylene tubing can be cut to a suitable length and one end heat-sealed to form a sterile, flexible culture container that is either a cylinder or an oblong bag (Baynes et al., 1979; Trotta, 1981). The culture vessel design is based on that used by SeaSalter Shellfish Company Ltd. (Farrar, 1975). Containers formed in this way can be strengthened by supporting them within a plastic-coated, steel-mesh frame (Fig. 11). Or, the cylinders can be suspended, with or without lateral support mesh, if the diameter of the bag is less than 30 cm and the height less than 200 cm.

Figure 11. Polyethylene bags used for continuous culture of microalgae. Bags are sealed top and bottom; one port in the bag introduces sterile medium and the other is used to harvest microalgae. The bags are supported by a cylindrical, steel mesh frame coated with vinyl (courtesy Bay Shellfish, Tampa, FL).

Continuous microalgal culture in polyethylene bags has several advantages: 1) a sealed container is less likely to become contaminated than a rigid container with an open top or lid; 2) bags do not require daily maintenance and cleaning; and 3) they cost less to install and use space more efficiently. Bags are the least expensive way of constructing large-scale culture vessels. Such containers can be used indoors with artificial illumination or outdoors in natural light. Polyethylene bags have a relatively short lifespan because the internal surface attracts culture debris and bacteria that reduce light penetration and are a source of contamination. At the end of a culture run, it is necessary to replace the bag.

Large-scale outdoor bag cultures are often positioned horizontally to maximize sunlight penetration (Fig. 12). Such large-volume systems are often used to induce

Figure 12. Outdoor culture of microalgae using polyethylene bags horizontally oriented to maximize illumination (courtesy Bay Shellfish, Tampa, FL).

multi-species blooms that are best suited for feeding juvenile shellfish in nursery systems or adult shellfish in broodstock systems, rather than for hatchery production. The rate at which a bloom develops is related to the species composition; the volume and cell density of the inoculum; the quantity, quality and duration of light; nutrient levels; and temperature.

Concentrating algal biomass

In most hatcheries, microalgae are fed in liquid form directly to the animal culture tanks. Recently, though, there has been an interest in concentrating algae to reduce the volume of microalgal culture water (and possible contaminants) introduced into culture tanks. The use of "algal paste" or concentrate has gained popularity because during periods of excess production, the concentrate greatly reduces the physical space required, can be refrigerated until needed, and can be diluted when used. However, the nutritional quality of the microalgae may be a concern if the concentrate is stored for extended periods. Although this concept is not new to aquaculture (Barnabe, 1990), algal paste in preserved or fresh forms recently has become commercially available

For small-scale harvests, filter screens or cartridge filters (1 to 5 µm) are effective. The concentrated cells are washed off with limited water and then used, refrigerated or preserved (Hoff and Snell, 2008). Chemical flocculation using natural organic agents such as gelatin, chitosan, and sodium alginate can be used to concentrate microalgae to feed benthic detritus feeders and crustaceans. Centrifugation is used to concentrate large-volume cultures. Continuous-flow centrifuges (e.g., Sharples Penwalt™) are used to concentrate microalgal cultures into paste. Both chemical flocculation and centrifugation have proven suitable in terms of efficiency and cell density when preparing concentrations for aquaculture feeds (Heasmann et al., 2000).

Advanced algal production systems

There are several new microalgal production systems on the market; they are collectively termed "AAPs" (Advanced Algal Production Systems) or photobioreactors. Photobioreactor systems can provide higher algal densities, more efficient space usage (a smaller footprint), continuous or semi-continuous production, longer production cycles with less contamination, and lower labor requirements (Ellis and Laidley, 2006). In general, three types of systems are under production: 1) tubular photobioreactors, 2) column or cylinder photobioreactors, and 3) flat-panel or plate photobioreactors (Tredici et al., 2009).

Closed photobioreactor systems have several advantages over conventional tank-based or pond microalgal productions systems. Still, they have limitations, such as overheating, oxygen accumulation, biofouling, and shear stress (Ellis and Laidley, 2006). Many of these designs are still under evaluation, so the reader is advised to review the specifications of these systems, including purchase and construction costs, operational efficiency, practical application to your production needs, etc. (Anderson, 2005; Tredici, et al., 2009).

Conclusion

Phytoplankton culture is a multi-faceted activity, and the task of designing a large-scale microalgal production system can be complex. The size, location, and engineering specifications should depend on the type of culture to be practiced (e.g., batch, semi-continuous, continuous), the site characteristics (such as water quality and temperature), illumination, the requirements of the target species, and production goals. These considerations are interdependent, and each should be treated as a part of the whole. The principles and methods described here represent only a few of many approaches to growing phytoplankton. The reader is advised to modify these guidelines as needed to suit specific hatchery conditions.

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United States Department of Agriculture
National Institute of Food and Agriculture

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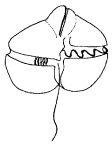
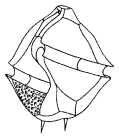
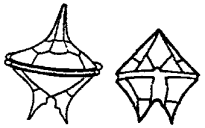
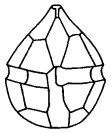
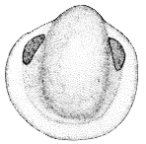
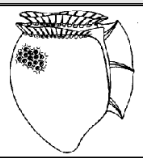
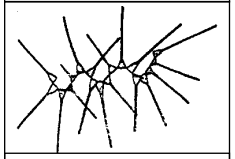
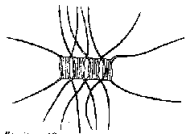
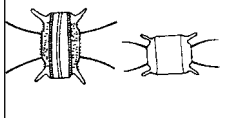
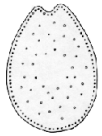
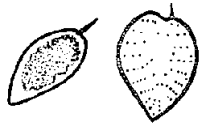
Target Species

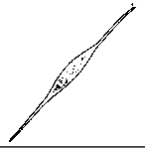
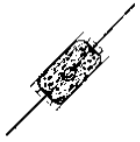
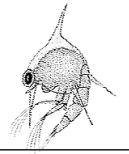
COMMON PHYTOPLANKTON KEY

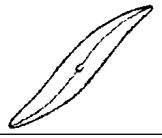
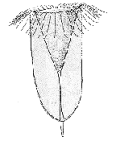
OTHER COMMON PLANKTON (non-phyto)

Alexandrium spp. **AL**	*Gymnodinium spp.* **GY**	*Gonyaulax spinifera* **GS**	*Protoperidinium spp.* **PT**	*Scripsiella spp.* **SC**	*Cocinodiscus spp.* **CO**	*Odontella spp.* **OD**	*Larval Clam* **LC**
							
25-46 µm	**24-50 µm**	**25-50 µm**	**50-95 µm**	**20-37 µm**	**40-500 µm**	**45-70 µm**	**Generally Large**

Dinophysis norvegica **DN**	*Dinophysis acuminata* **DA**	*Dinophysis tripos* **DT**	*Asterionellopsis spp.* **AS**	*Chaetoceros spp.* **CH**	*Chaetoceros socialis* **CS**	*Biddulphia spp.* **BD**	*Rotifer spp.* **RO**
							
48-80 µm	**40 - 50 µm**	**40 - 120 µm**	**30-150 µm**	**10 - 53 µm**	**3-15 µm**	**60 - 160 µm**	**Generally Large**

Prorocentrum lima **PL**	*Prorocentrum micans* **PM**	*Ceratium fusus* **CF**	*Ceratium lineatum* **CL**	*Ceratium longipes* **CP**	*Dictyocha spp.* **DO**	*Fragilaria spp.* **FR**	*Pollen Grain* **PG**
							
31-47 µm	**35-70 µm**	**200-540 µm**	**100-130 µm**	**150-250 µm**	**10-45 µm**	**10 - 70 µm**	**Generally Large**

Pseudonitzschia **PS**	*Thalassionema spp.* **TA**	*Thalassiosira spp.* **TL**	*Nitzschia spp.* **NZ**	*Skeletonema spp.* **SK**	*Ditylum spp.* **DM**	*Leptocylindrus spp.* **LP**	*Crab Zoea* **CZ**
							
64-117 µm	**16 - 90 µm**	**12-39 µm**	**60 - 125 µm**	**2-21 µm**	**80 - 130 µm**	**30 - 75 µm**	**Generally Large**

Species Name **CODE**	*Rhizosolenia spp.* **RH**	*Gyrosigma spp.* **GY**	*Navicula spp.* **NV**	*Melosira spp.* **ML**	*Guinardia spp.* **GN**	*Eucampia spp.* **EU**	*Tintinnid spp.* **TN**
(Guide to using key) *illustration of organism*							
Size Range (in µm)	**25-57 µm**	**110 - 175 µm**	**32-49 µm**	**10-50 µm**	**60 - 160 µm**	**10-33 µm**	**Generally Large**

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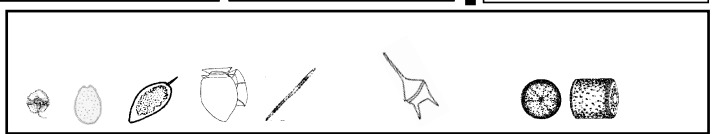

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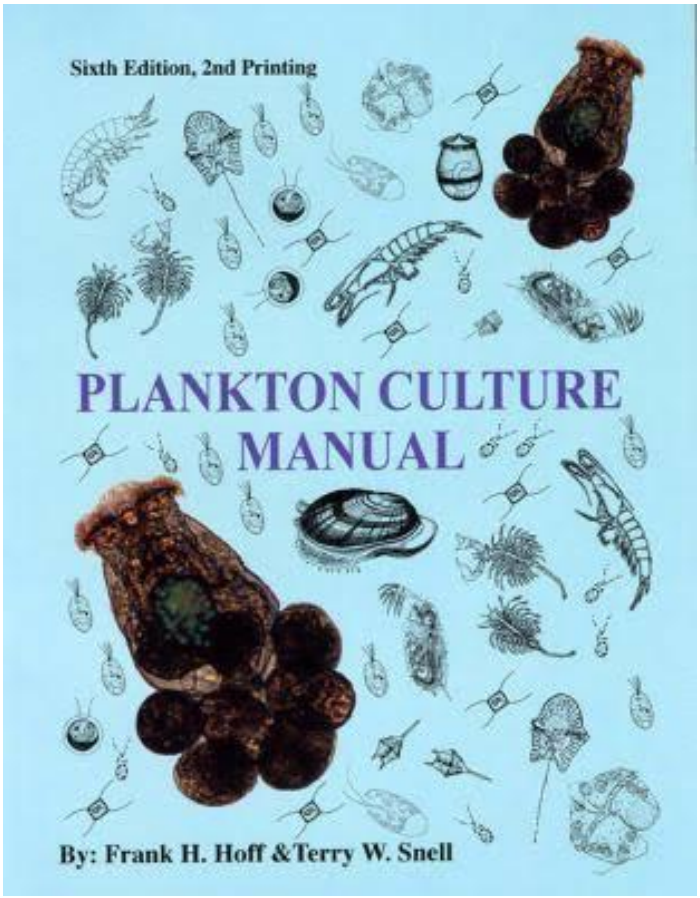

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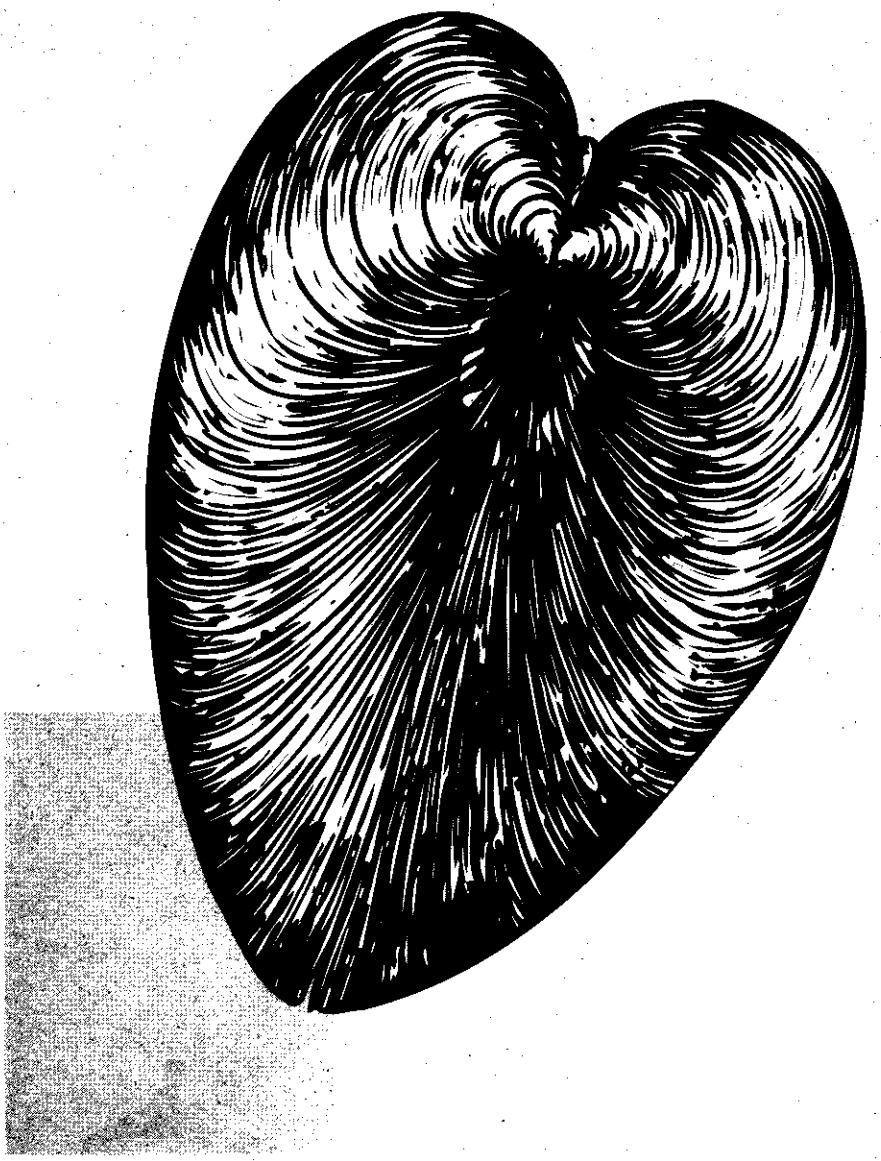
PLANKTON CULTURE MANUAL 6TH EDITION

By Frank Hoff & T. Snell. Published by Florida Aqua Farms (6th edition). Expanded version includes identification of microalgae used in aquaculture manual. Manual now includes use of microscope, slide preparation, measuring cells, stains, key to genera, description of cells variations, contaminants in cultures and photos. Provides updated step by step instructions for culturing phytoplankton (microalgae) and zooplankton including rotifers, adult brine shrimp, ciliates, copepods, daphnia, Oyster and clams veligers, amphipods, mysid shrimp and microworms. Easy to read but very informative. We have provided technical data and illustrations to support our instructions in a clear understandable format. Sections include trouble shooting and reasons for failure. Includes simple culture methods and more intensive, large scale methods and designs. This manual serves as a companion to our Conditioning, Spawning Manual below. Extensive references, 8.5” x 11” format, 183 pages, 84 illustrations, 21 tables.

A MANUAL FOR THE CULTURE OF THE HARD CLAM *MERCENARIA* SPP. IN SOUTH CAROLINA



by

Nancy H. Hadley, John J. Manzi, Arnold G. Eversole, Robert T. Dillon, Colden E. Battey, Nancy M. Peacock

Table 10.
Approximate size/volume relationships of post-set and juvenile clams

Size (Length, mm)	#/ml
0.3	99,000
0.4	42,000
0.5	21,000
0.6	12,000
0.7	8,000
0.8	5,000
0.9	4,000
1.0	3,000
1.5	800
2.0	300

small volume of clams (e.g. 0.5 ml) is measured in a graduated, conical bottom centrifuge tube, using the same technique as for determination of packed volume. The clams are then transferred to a petri dish for counting under a dissecting scope. If clams are too numerous to count all of them, a gridded petri dish can be used. Disperse the clams as evenly on the petri dish as possible and count enough grids to total 1/10 of the total dish area. From this the total number is extrapolated. Alternatively, pile a known volume (e.g. 0.5 ml) of post-set on a small dish and divide in half. Remove one half and divide the remainder in half. Repeat this procedure until the number remaining is ~100–200. (Make sure to keep track of the number of divisions in order to calculate the total number). To facilitate counting, the clams should be rinsed with freshwater and the water gently decanted until fairly dry. (In seawater the clams will crawl around the dish.) Clams will wash around rather easily if the water is not removed from the dish, making counting very difficult.

ALGAL CULTURE

The final major process in the hatchery is food production. Although numerous substitutes have been tried, no adequate replacement for live phytoplankton as a larval and post-set diet has been found. Therefore, culture to produce large quantities of suitable phytoplankters is an integral part of a hatchery operation.

This section describes the "Milford" method of phytoplankton production. Most of the information given here is derived from Guillard (1983). Other methods of algal culture are described in Castagna and Kraeuter (1981), Ogle (1982) and Castagna and Manzi (1989).

The method described here is a sequential culture method utilizing small cultures to inoculate successively larger cultures until the final production stage is reached **(Fig. 14)**. The MRRI system utilizes 4 levels of culture: stock cultures (25 ml test tubes); intermediate cultures (500 ml flasks); carboys (4–20 liter glass or polycarbonate jugs); and final stage production (200 liter Kalwall® tubes). Test tubes and flasks are kept free of bacteria and other contaminants and provide a long-term source of clean algal cells for inoculating larger

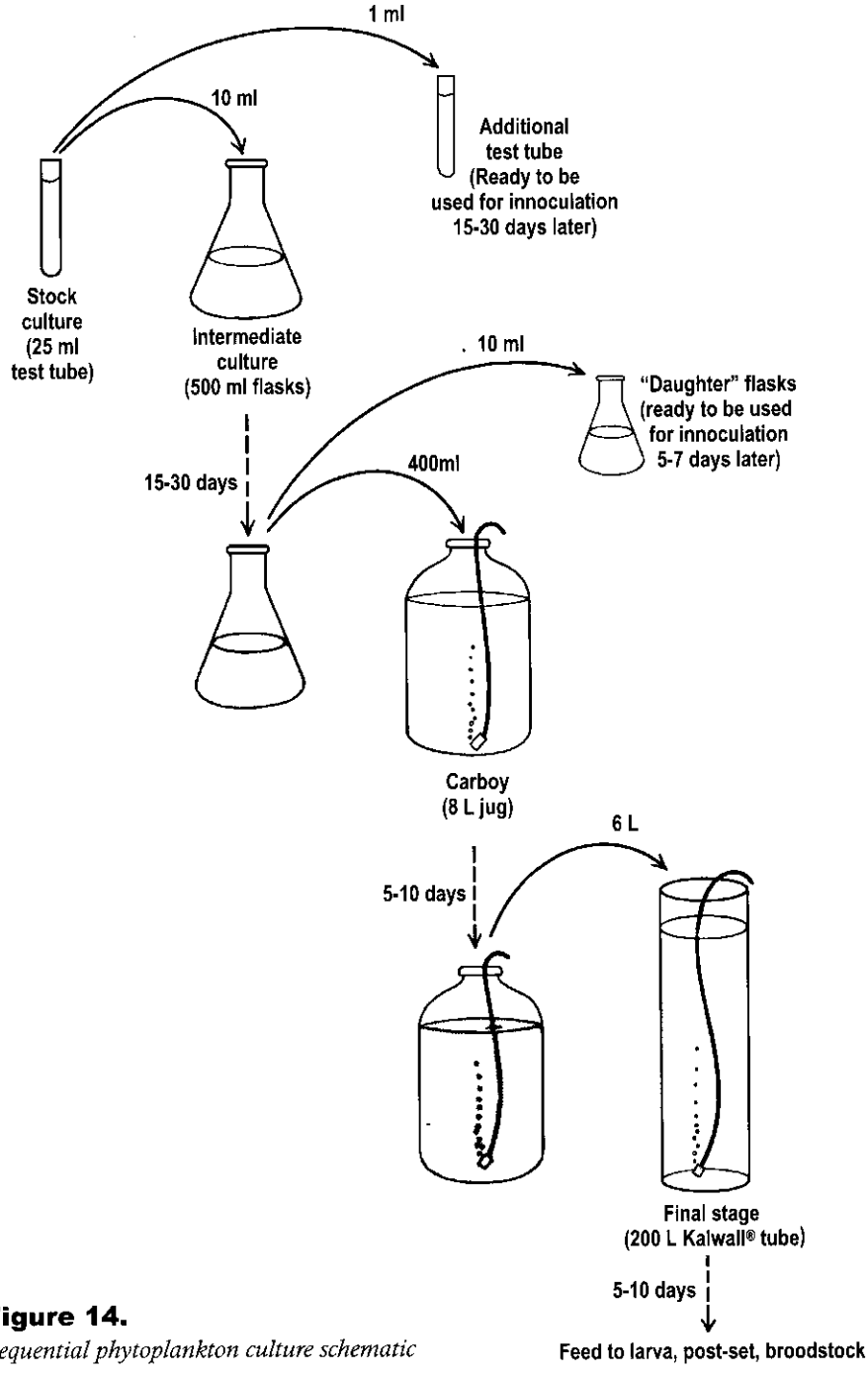


Figure 14.
Sequential phytoplankton culture schematic

cultures. The larger cultures by their very nature are prone to contamination and are therefore grown for as short a time as possible prior to use. The entire process from the smallest culture to the harvest stage requires 15–30 days.

A typical culture cycle starts with inoculating one or more flasks from a stock test tube. Additional test tubes are inoculated at the same time. After 5–10 days, the flasks are used to inoculate daughter flasks and a carboy. The carboy is allowed to bloom for 5–10 days and is then used to inoculate a Kalwall®. After 5–10 days the Kalwall® is harvested and fed to the clams.

Sufficient algae must be cultured to meet the current feeding needs, inoculate for future needs, and guard against unexpected "crashes". Depending on algal culture densities, broodstock conditioning may require 30–50 liters of algae a day for about 50 broodstock. One million larvae will require 1–10 liters a day, one million post-set 10–100 liters per day. There must always be enough carboys and Kalwalls® on line to accommodate this feeding rate and to provide backups in case of crashes. Cultures should be at high densities (3–5 million algal cells/ml) before feeding. This will allow you to add enough algal cells without adding excessive algal media.

Starter phytoplankton cultures are available from a number of sources (see Appendix D). Many institutions maintain cultures for research purposes and several private companies market some of the commonly used species. A list of algal collections is contained in Miyachi et al (1989). Established hatcheries are often willing to share their stock cultures, although these may not always be pure. If you have the time, patience and necessary equipment, you can isolate your own algae from raw seawater, but most hatcheries just obtain starter cultures from other sources.

Culture environment

A number of factors must be considered regarding the physical environment for cultures: types of culture vessels; light; temperature control; aeration and pH control.

Culture vessels

Culture vessels must be of appropriate size and material. The choice of vessel depends on several factors, such as size of autoclave, height of shelving, arrangement of lights, budget restrictions, etc. Dimensions of culture vessels will determine the arrangement of the algal culture areas (or the available space may be determine the size vessel to purchase). Tubes, flasks and carboys should be autoclavable. Typical culture vessels are 15–50 ml glass test tubes, 500–2000 ml Erlenmeyer glass flasks, and 4–20 liter (1–5 gallon) clear glass or polycarbonate carboys or jugs. Stoppers for each type of vessel are also necessary: screw-caps for tubes, disposable plugs or screw-caps for flasks and cotton plugs for carboys. An ample supply of tubes, flasks, carboys and stoppers should always be available for transfers and to compensate for inevitable breakage.

Carboys are used to inoculate larger cultures, usually the final stage in the culture process. One of the easiest vessels to use for this purpose is a transparent fiberglass cylinder available commercially in a range of sizes and shapes. The transparent fiberglass may be purchased in sheets from solarium suppliers to custom-build your own containers. The cylinders were marketed for several years as Kalwalls® but are now called Sunlite® tubes. Because of its familiarity, the term Kalwall® will be used in this manual.

Large hatcheries may include another culture stage beyond Kalwalls®. This is usually large, shallow (<1 m) fiberglass tanks which hold 1000 or more liters. These are inoculated with one or more Kalwalls®.

Light requirements

Rapid growth of the phytoplankton cultures, particularly in the carboy and Kalwall® stages, is essential and can only be accomplished with supplemental lighting. Fluorescent tubes are the most widely used source of light for production of algae. These can be grow-light bulbs, but algae will grow well with less expensive bulbs, commonly described as "cool-white", "daylight" or "natural". Using a mixture of these (e.g. equal numbers of daylight and natural) works well. For Kalwalls® and particularly for fiberglass tank cultures, 250–400 watt metal halide lamps are widely used. These are generally marketed for warehouse or outdoor lighting applications and are available in a variety of housings.

Not all marine algae tolerate continuous illumination, but most species commonly raised as bivalve food will. Although there is no use supplying more light energy to a culture than will result in increased yield, larger cultures (Kalwalls® and tanks) are often light-limited. The saturation level for yield will depend on the volume and density of the culture. Consult Guillard (1983) for more detailed information on saturation levels and light intensity.

Temperature control

Although marine algae will grow at room temperature (23–28°C = 73–82°F), cooler temperatures (19–20°C = 66–68°F) will help to inhibit bacterial contaminants. Because the artificial lighting produces a considerable amount of heat, the algal culture room will have to be air- conditioned most of the year. Most marine phytoplankton cannot tolerate temperatures above 35°C (95°F).

A smaller controlled environment chamber or incubator, with temperature and light control, is useful for stock cultures. Stock cultures should be kept in a separate area from carboys, to reduce the chance of contamination and to provide backup cultures in the event that carboys become contaminated or environmental controls in the main culture area fail. Stock cultures should be maintained at 15°C (59°F) to retard growth and inhibit bacteria.

Aeration and CO_2 addition

Aeration of cultures serves to keep algae in suspension, to supply the carbon needed for plant growth and pH control, and to strip O_2 from the culture media, preventing supersaturation. Carboy and Kalwall® cultures always require aeration, which should be gentle during the first day or two after inoculation and then increased in rate as the culture grows. Flasks and tubes, however, are never aerated, as the chance of contamination is too high. They should be shaken by hand once a day.

Optimum pH levels for phytoplankton are between 7.0 and 8.5. The pH of coastal seawater is usually between 7.0 and 8.0. As the phytoplankton grow, they utilize carbon in the seawater, causing the pH to increase. If no measures are taken to counteract this, the pH in a rapidly growing culture can easily exceed 9.0. High pH values inhibit phytoplankton production. Aeration has some ameliorating effect on pH, but air alone cannot effectively control pH in very dense cultures. Increasing the CO_2 content of the aerating gas makes pH control possible. Adding commercial-grade CO_2 for 30–45 seconds each hour through the air distribution system is usually adequate to maintain pH within the desired range. This can be accomplished with a cycle timer and a CO_2 cylinder equipped with a regulator. The output is sent directly into the air distribution line. The regulator pressure should be adjusted to a level which just exceeds the air pressure in the distribution line. If the regulator is set too high, the pH will be driven too low. Only enough CO_2 should be added to reduce the pH to 7.0–7.5. At the end of an hour, it will have risen to about 8.0–8.5.

The most common cause of contamination in algal cultures is the air distribution system, which is typically moist with condensation and provides a good environment for bacterial growth. In-line bacterial filters placed on the airline feeding each individual culture container will greatly reduce the contamination rate. If your budget is tight, acceptable filters can be made by stuffing pipettes or plastic tubes with cotton. Most air pumps have a built-in filter which should be cleaned regularly.

Routine for production of algae

Production and maintenance of algal cultures demands a routine which depends on the species of algae grown and the quantity of algae required. The most important point is to make transfers (inoculations) before half the life span of the culture has passed. In most cases, transfers should be made at least once a week. Stock cultures should be transferred at least once a month. A weekly routine of transfers allows sufficient growth time for algae which is well within the life span limit.

Preparation of culture medium

Seawater

Natural seawater of suitable salinity (>25 ppt) is the most satisfactory medium for growing phytoplankton in large quantities.

For very small quantities, it may be possible to use artificial seawater. Prior to use, seawater must be filtered to remove native phytoplankton, zooplankton, detritus and silt. A 25 µm filter bag will remove most zooplankters. To remove phytoplankters and very fine silt, it is necessary to further filter the water through a 1 µm filter bag or cartridge. It is often necessary to settle the water between the gross filtration (25 µm) and fine filtration (1 µm) steps. Otherwise the heavy silt load will cause frequent filter clogging. Most of the silt will settle out in 24–48 hours. For test tubes, flasks, and nutrient stock solutions, a final filtration step may be included to remove bacteria. This will require a vacuum source and a special vacuum filtering apparatus which holds a glass fiber filter disk **(Fig. 15)**. Only small amounts of water (usually up to a liter) are filtered at a time and the filter may need to be changed between batches. A glass-fiber filter in the 0.2–0.45 µm range removes all bacteria.

Sterilization

The culture containers (tubes, flasks, carboys, Kalwalls®) and the culture water must be sterilized prior to introducing the algal inoculant. Improper or inadequate sterilization will result in contaminated cultures which are not useable in the hatchery. After inoculation, every effort is made to keep tubes and flasks sterile; carboys and Kalwalls® are kept as uncontaminated as practicable.

Tubes, flasks and small carboys are usually sterilized by heat and pressure, using an autoclave, pressure cooker, or pressure canner. A microwave can also be used to sterilize small vessels. The culture water is sterilized simultaneously. Clean containers are partially filled (½–¾ capacity) with seawater which has been filtered as described above. If the containers are too full they may boil over during the sterilization process. Nutrient media (see below) is added to test tubes prior to autoclaving. Sodium silicate, if needed, is added to test tubes and flasks prior to autoclaving. The prepared containers of seawater are closed with a screwcap (not completely tightened) or a foam or cotton plug covered with aluminum foil. They are then autoclaved for 15 minutes at 250°C or pressure-cooked following manufacturer's instructions for canning. After sterilizing, the culture media must be cooled to room temperature prior to use. Transfer pipets should be sterilized in this manner also. They should be wrapped in autoclavable plastic or aluminum foil or placed in a sealed autoclavable container.

Larger carboys and Kalwalls® may be sterilized with chlorine. After thorough cleaning, the containers are filled with 1 µm bag- or cartridge-filtered seawater and household bleach is added at the rate of 0.5 ml/liter. Airlines and airstones are placed in the containers so that they are sterilized at the same time. The container is chlorinated for at least 30 minutes. After 30 minutes or more of sterilization, sodium thiosulfate (0.05 g/L) is added to neutralize the chlorine. Allow at least 30 minutes for the neutralization and check with a chlorine test strip or test kit before adding nutrient media.

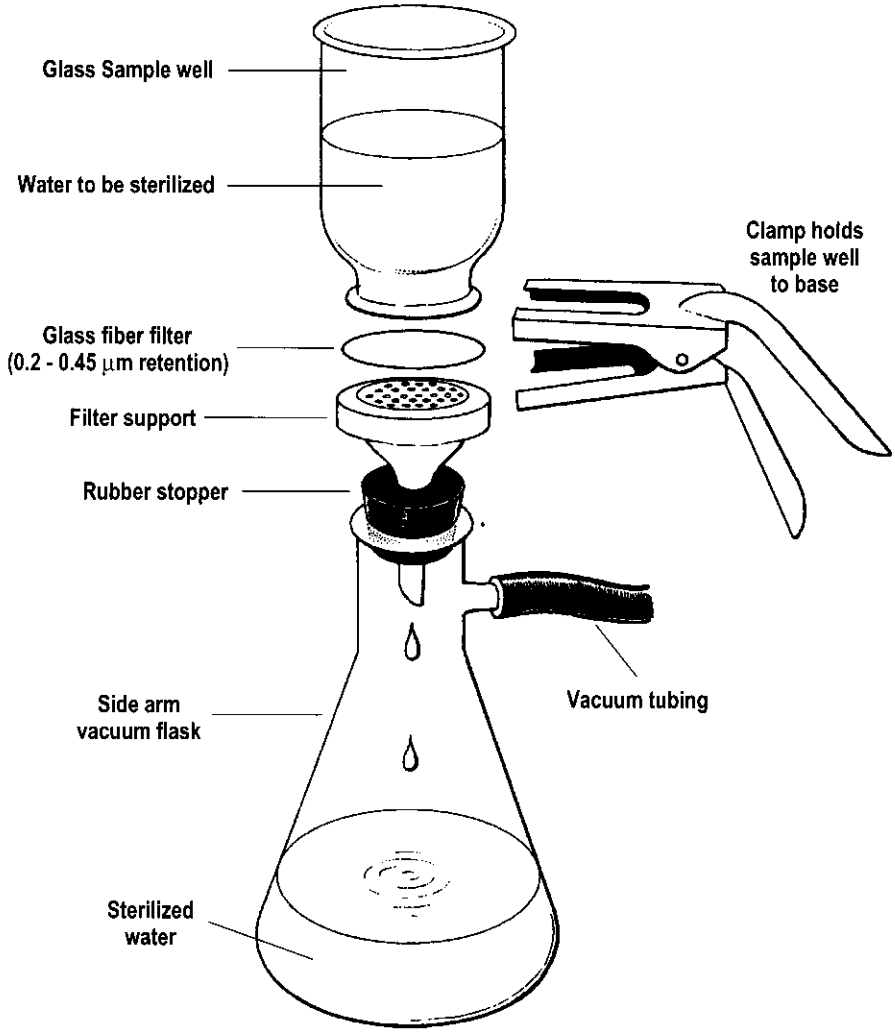


Figure 15.

Vacuum filtration apparatus for cold sterilization of seawater

Nutrients

Seawater alone does not have enough nutrients to support dense phytoplankton blooms and must be enriched with appropriate nutrients prior to inoculation. The most commonly used culture medium is referred to as "f/2". Some hatcheries mix their own nutrients "from scratch". If you wish to explore this alternative, consult Guillard (1983). Mixing your own allows you to vary the concentration of selected nutrients. However, many different stock solutions must be maintained and it is much easier to buy pre-mixed nutrient packages available from several vendors (see vendor list in Appendix C). These are available in liquid or dry form, with instructions for diluting to achieve f/2 strength. In addition to the basic nutrients, diatoms require silicate for growth, so this must also be added. It is usually mixed and stored separately from the other nutrients to avoid precipitation. For short-term culture purposes, household plant fertilizer is an adequate substitute for the more expensive specially formulated media.

Nutrients are stored as concentrated stock solutions. A small aliquot can then be added to a larger volume of seawater to produce the desired strength medium. Stock cultures (test tubes) are maintained in dilute media (e.g. f/4 = half the strength of f/2) to retard growth, while other stages are generally raised in f/2. It is desirable to make separate stock solutions for small and large cultures. Convenient concentrations might be 25X (25 times as concentrated as f/2) for tubes and flasks and 1,000X (1,000 times as concentrated as f/2) for carboys and Kalwalls®. To achieve f/2 strength in the final media, 1 ml of 25X solution would be used for each 25 ml in a flask. For tubes, which should be maintained in f/4, 0.5 ml of the 25X solution would be used for each 25 ml of media. One ml of 1,000X stock would be added to each liter in a carboy or Kalwall®. Any convenient stock concentration is acceptable, as long as the final media concentration is correct. Make up relatively small quantities of stock solution at a time, so that it will be consumed in about 30 days. Using a stock solution over a longer period of time may result in contamination. If you purchase a liquid formulation in a large container, it is advisable to transfer it to several smaller containers.

To prepare the stock solutions, select an appropriate storage container (autoclavable, glass or plastic, with screw cap) and clean with laboratory-grade detergent. If there are any precipitates on the glass, acid-washing may be needed. Choose a container which will be almost filled by a 30 day supply of your stock solution. For instance, for flasks and tubes a 1 liter container is appropriate, while a 4 liter container might be needed for carboys and larger cultures.

Fill the container with finely filtered seawater. If possible it should be vacuum filtered through a glass-fiber filter with retention capacity of 1.2 µm or smaller. Cover the container and sterilize as described above. When cool, add the appropriate amount of pre-mixed nutrients

to obtain the desired stock solution. Pre-mixed nutrients which are in liquid form may be cold-sterilized prior to addition by vacuum filtering through a very fine filter, such as a 0.2 μm membrane filter. Stock solutions made with dry-packed nutrients may be cold-sterilized after the solutions are made. We have experienced little trouble with nutrient contamination and do not routinely cold-sterilize our stock solutions.

Silicate stocks are prepared in distilled water, rather than seawater. The desired final concentration is 15–30 mg/liter of sodium metasilicate. It is convenient to make two stock solutions: 100X strength (1.5–3.0 g/liter) for flasks and tubes and 1000X strength (15–30 g/liter) for carboys and Kalwalls®. Use 1 ml of the 100X stock for each 100 ml of final media for flasks and tubes. Use 1 ml of 1,000X for each liter of final media for carboys and larger cultures. The silicate solution should be autoclaved.

Store the stock solutions in the refrigerator, tightly capped. Remove stock solution as needed with a sterile pipet, or by pouring. Open as seldom as possible to avoid airborne contamination. If a large quantity is prepared at one time, it may be advisable to store in several small containers to reduce the number of times a container is opened. Label and date the stock solutions and discard when a month old.

For stock cultures (test tubes), nutrients may be added prior to autoclaving the culture water. For other culture levels, add the nutrients to the seawater after it is sterilized, immediately prior to inoculation. A precipitate forms in f/2 medium upon autoclaving, particularly in large volumes of water. This is avoided by adding the medium after autoclaving. Additionally, some of the vitamins may be denatured by the heat of autoclaving. Stock cultures, which do not need to grow rapidly, do not appear to be adversely affected by any loss of vitamins, but the production cultures will probably perform better if the vitamins are not autoclaved.

Inoculation and culture

Stock cultures

Stock cultures (test tubes) are used for long-term maintenance of uncontaminated algal cells. Stock cultures should be kept isolated in a carefully controlled environment. Temperature should be maintained at 15°C ± 1°C and appropriate lighting (preferably designed for plant growing) must be provided. Constant illumination may be provided, or lighting may be timed to simulate a diurnal cycle. Tubes should be agitated daily to keep the cells in suspension. Media for stock cultures is usually dilute ("f/4" or less) to retard growth. If full strength media is used, the stock cultures will grow too rapidly and will exhaust the nutrient supply unless more frequent transfers are made. Stock culture tubes are opened only when transfers are made.

At least once a month, new stock cultures should be inoculated from the old ones.

This assures that healthy stock cultures are always available in case other levels of production become contaminated. After inoculating "daughter" test tubes, the remainder of the stock culture is used to inoculate one or more flasks. This rejuvenates the flask culture level, which is more prone to contamination than the tubes. If possible, transfers should be made under a hood with an ultraviolet lamp, which kills bacteria. At the very least, they should be made in a clean, dust-free area, with no drafts.

Preparation of culture tubes has already been described. Remember to add nutrients ("f/4" or less) to the tubes prior to sterilizing. Several new tubes may be inoculated from one older tube. This will provide additional emergency stocks in case a catastrophe wipes out all your flasks. One or two flasks (depending on the size of the tubes and flasks) should also be inoculated at the same time.

To inoculate, gently agitate a stock culture tube to evenly distribute the algal cells. Open the stock culture tube and use a sterile pipet to transfer 1 ml to each new tube, immediately capping the inoculated tube. Pour the rest of the stock culture into a prepared flask(s). Use about 10 ml to inoculate a 500 ml capacity flask containing 250–350 ml of media. Label the newly inoculated cultures with the species identification and the date of inoculation. Add the appropriate amount of nutrients from the stock solution to the flask (nutrients are already in the tubes). Repeat this process for each stock culture tube to be transferred. Make sure to use a new sterile pipet for each tube.

Flask cultures

Erlenmeyer flasks are commonly used as the first level of the production process. A 500 ml capacity flask containing 250-350 ml of media will produce enough inocula for a 4–8 liter (1–2 gal) carboy (also ½–¾ full). If larger carboys are used, you may wish to use larger flasks. Flasks up to 2 liter capacity are manageable for this stage. The flasks may have screw caps or may be stoppered with disposable foam plugs or homemade cotton plugs. Flask cultures are grown in a well-lighted area, preferably provided with special plant-growing lights. Although the algae will grow at room temperature (23–28°C), cooler temperatures (19–20°C) will inhibit bacterial growth. Flasks are not aerated but should be agitated daily to resuspend algal cells.

Glassware and seawater for flask culture are sterilized as described in the previous section. Flasks may be prepared in advance and stored (tightly covered) in a clean cabinet until use.

On a weekly basis, flasks are used to inoculate new flasks and carboys. A single flask can inoculate 1 or 2 new flasks and a carboy. If more flasks are available than needed for inoculations, they may be allowed to grow for a second week. Flasks more than two weeks old should be discarded. To inoculate, select a flask with a dense culture. Agitate the flask to evenly mix the algae. Use a sterile pipet to transfer 10–20 ml of algal culture to each new flask. Use the remaining amount to inoculate a carboy (see below). Add

nutrients to the new flasks, cap, and label. On a monthly basis, and whenever there are signs of contamination, flasks should be inoculated from stock culture tubes, as described previously. These will take longer to bloom than flasks inoculated from other flasks.

Carboy cultures

Carboys are glass or clear plastic containers, usually jug-shaped, with a capacity of 4–20 liters (1–5 gal). Carboy cultures may be grown at cool room temperature (23–28°C) but bacterial contamination will be retarded if they can be kept cooler (19–20°C). They must be supplied with artificial lighting and aeration. They should be located in a low-traffic area to prevent breakage.

Carboys are sterilized prior to use by autoclaving or chlorination as described previously. After sterilization, nutrients and sodium silicate from stock solutions are added. The carboy is then inoculated with algal culture from a flask by simply pouring the contents of the flask into the carboy. The carboys will reach harvest density more rapidly if more inocula is used. If 250 ml is used to inoculate 2.5–3.5 liters of seawater, it will be ready for use in 3–5 days. If the same amount is inoculated into 5–7 liters, it may take 6–10 days to grow. The longer the culture must be grown, the more likely that it will become contaminated. For larger carboys, two flasks of the same species may be used as inocula.

Carboys require aeration. Use clean airlines and a clean, self-weighting airstone. Cover the top of the carboy with a sandwich bag, inverted plastic beaker, or other protective covering to prevent airborne contamination. Use an in-line bacterial filter or cotton-plugged tube between the air source and the carboy. Do not aerate too vigorously—a slight stream of bubbles is adequate. CO_2 addition is optional.

Observe carboys daily to make sure they are properly aerated and to assess the condition of the algae. If the culture is not noticeably denser after three days, it should be discarded. Cultures are usually ready to use as Kalwall® inocula after 5–7 days. If not used within 2 weeks they should be discarded.

Kalwall® cultures

Kalwalls® are clear fiberglass cylinders available commercially in several sizes. They are currently marketed as Sunlite® tubes. The most commonly used sizes are 80 and 200 liter. These may be fitted with a valve for ease of draining. Cultures are grown at cool room temperature with intense artificial lighting, aeration and addition of CO_2 to control pH. Kalwall® cultures usually require 5–7 days to become dense enough to be useful as food. Cultures more than 14 days old should be discarded.

Kalwalls® are sterilized with bleach as previously described. After neutralization with sodium thiosulfate, stock nutrients are

added. The culture is then inoculated with the entire contents of a carboy. The amount and density of the inoculant will effect the growing time prior to harvesting the Kalwall®. A 150 L culture inoculated with 3 liters from a dense carboy will require 4–6 days to reach harvest density (3-5 X 10^6 cells/ml). The Kalwalls® must be well aerated and should be covered with a lid or plastic bag to exclude air-borne contaminants. An in-line bacterial filter should be used on the airline. The pH should be checked regularly, either with pH strips, a hand-held meter, or a pH probe suspended in a Kalwall® and connected to a remote meter.

Kalwall® cultures are more prone to contamination than the previous culture stages. Signs of contamination are foam on the surface of the water, abnormal color change (e.g. from brown to greenish), clumping of algal cells, slimy growth on the airline, or failure of the culture to bloom in a reasonable period of time. Suspect cultures should be examined microscopically. Contaminated cultures should be discarded immediately.

Cleaning practices

All glassware and equipment used for algal culture should be cleaned with laboratory detergent and hot water before use. Additionally, glassware may need to be dipped in dilute muriatic acid occasionally to remove mineral deposits. Culture vessels and transfer pipets must be sterilized. Autoclaving equipment (e.g., vacuum hoses, filtering rigs, etc.) is also an excellent idea to prevent unwanted contamination.

After each harvest, Kalwalls® should be scrubbed with laboratory-grade detergent and hot water. The slightly irregular surface of the fiberglass allows deposits and growths to build up. To prevent this, the Kalwall® is soaked with muriatic acid (~50 ml in 200 liters of water). Make sure a cover is on the cylinder while it is soaking to contain fumes. After soaking, drain and rinse well with freshwater.

CAUTION: Use gloves and eye protection when handling acid. Muriatic acid and chlorine bleach combine to release an extremely toxic gas. Be very cautious not to use these chemicals in the same area or discard them in a common drain!

Determining algal cell concentrations

Kalwalls® are ready to harvest when they reach a density of 3–6 million cells/ml. There are several ways to determine algal density, including a modified secchi disk, fluorometry, spectrophotometry, Coulter® counting, and counting with a hemocytometer. An experienced technician can usually estimate algal density fairly accurately by observing the color of the culture. However, all of these methods require that the algae be manually counted periodically for calibration. This is done with a hemocytometer and a compound microscope.

A hemocytometer is a special microscope slide originally used in the medical field for counting blood cells **(Fig. 16)**. The slide is constructed to evenly disperse a

very fine film (0.1 mm deep) of the solution to be counted under a special coverslip. Two specially marked grids form counting areas and are surrounded by a well. The hemocytometer and coverslip must be very clean in order to work properly. Clean both with 10% isopropyl alcohol prior to use and wipe dry with a laboratory grade tissue or lens paper. Be particularly careful to remove any fingerprint grease. Lightly dampen a fingertip with saliva and touch the raised wall - not the counting surface. Immediately place the coverslip on the slide so that its edges rest on the dampened spots, which hold the coverslip in place.

A small (e.g. 100 ml) sample of the algal culture to be counted is procured in a beaker. If the algae is a motile species (e.g. *Isochrysis*), it must be killed prior to counting. An effective way to do this is with a drop of Lugol's solution. Lugol's solution is made by mixing 6 g of potassium iodide and 4 g of iodine in 10 ml of seawater. Lugol's solution is also available ready-mixed from most scientific houses. Non-motile species such as most diatoms do not need to be killed prior to counting.

A long-tipped disposable Pasteur pipet is used to load the hemocytometer. Before drawing the sample, mix the algal cells by submerging the pipet and blowing gently through it. Then allow the pipet to draw a small sample by capillary action. Do not suck algae into the main body of the pipet. Hold the pipet at a 45° angle and gently touch the tip to the "V" slot at one end of the coverslip. A small amount of sample should immediately be sucked under the coverslip by capillary action, completely covering the silvered counting grid. Repeat this process to load the other counting grid. If the sample spills over into the well area, empty the slide and start over. If the sample does not fully cover the silvered grid, reapply. The time from drawing the algal sample to filling the chamber should be short to minimize settling of cells in the pipet.

Place the loaded hemocytometer on a compound microscope and set the magnification to 100X (10X ocular and 10X objective). Move the slide around until you locate one of the counting grids **(Fig. 16)**. Typically, the slide is divided into 5 counting areas: four corner squares and a central square, each of which contain exactly 0.0001 ml of the sample. These counting areas are subdivided: 16 small squares in the corners and 25 small squares in the central region. (If your hemocytometer has a different type of counting grid, refer to the instructions with the slide). If all algal cells in any one of these five regions are counted, that count multiplied by 10,000 is the number of cells per milliliter in the original sample. Since algal cultures are usually very dense, a subsample of the smaller squares may be counted. For instance, 5 of the 25 small squares in the central region may be counted. This number is then multiplied by 5 (to get the total in the entire central region) and by 10,000 to yield the number per milliliter. When algal concentration is <500,000 cells/ml, the cells in all five counting regions should be counted and that total multiplied by 2,000 to yield the number per milliliter. When densities are

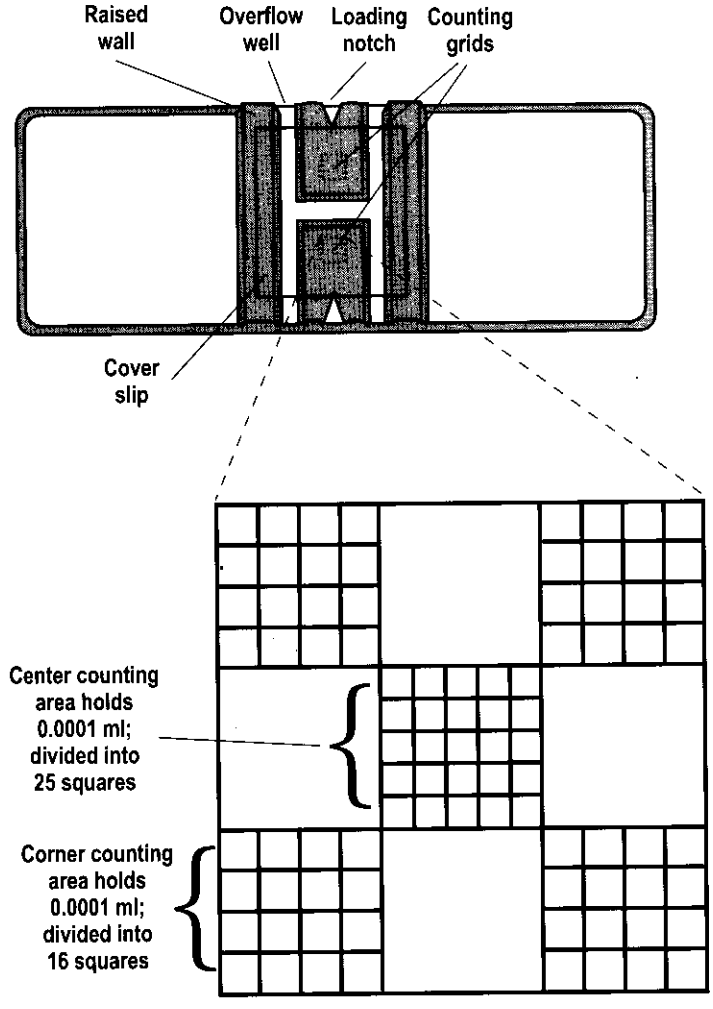


Figure 16.
Hemocytometer slide for counting phytoplankton

very low (<100,000), all cells in all five regions of both counting grids may be counted and that total multiplied by 1,000.

Troubleshooting

Most algal culture failures are due to contamination. Contaminated cultures are characterized by foaming of the surface, clumping within the culture, unusual coloration, or failure of the algae to reach appropriate densities in a reasonable period of time. If any of these symptoms are observed, the culture should be examined under the microscope. A healthy culture will be very clean—all the algal cells similar in appearance, no extraneous organisms (e.g. ciliates) or foreign objects (e.g. hair, dust), and no clumping of algal cells. If the species is a flagellate (e.g. *Isochrysis*), the cells should be moving. If there are any other organisms (usually ciliates) present, or if the algal cells are clumped (a sign of bacterial contamination), the culture should be discarded. Some possible sources of algal culture problems and corrective measures are listed in **Table 11**.

Choice of phytoplankton species to culture

There are many marine phytoplankton which could be cultured for use in shellfish hatcheries, but relatively few are in common use. The choice of algal species to culture depends on a number of factors, including availability, food value, and ease of culture. Some sources of starter cultures are listed in Appendix D. There are many reports of feeding studies in the literature (e.g. Davis and Guillard 1958, Epifanio 1979, Enright et al. 1986, and Tan Tiu et al. 1989) which can be consulted to determine whether a species is a suitable food source. Probably the most commonly cultured species is *Isochrysis galbana*, which is often used as the control diet in feeding studies. It is desirable to culture two or more types of algae in order to provide a nutritionally complete diet for the clams. Usually one of the species cultured is a diatom. Some of the commonly cultured species are described in Appendix D. An algal species which grows well in one hatchery may not in another, so it may be advisable to start with several species and determine which grow best in your facility. Tahitian *Isochrysis galbana*, *Chaetoceros muelleri*, and *Chaetoceros gracilis* have proven reliable in the culture system at MRRI.

GENETIC CONSIDERATIONS FOR HATCHERY MANAGERS

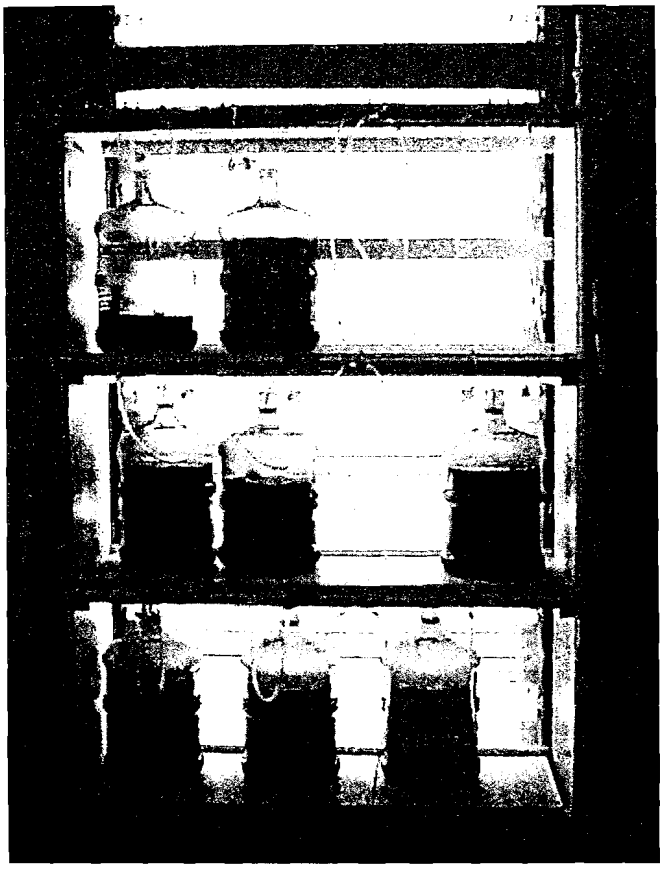
Some hatcheries have elaborate breeding programs to improve stock performance. If your hatchery needs are small (e.g. you only need one or two spawnings per year to produce the required seed) it may not be necessary to have an elaborate breeding program. Even small hatcheries, however, usually choose broodstock from their own seed once it reaches maturity. For this reason it is very important to follow certain protocols to insure the genetic "health" of your stocks. The most important of these is to include enough genetic variation in each spawning. If genetic

Table 11.
Sources of algal culture problems and corrective measures.

Source	Corrective measures
Water not properly sterilized	Review procedures Check operation of autoclave Verify chlorination with check strips or kits
Aeration supply contaminated	Add/replace in-line air filters Sterilize airstones Sterilize distribution pipes
Subcultures contaminated	Examine flasks and tubes for contamination Review inoculation procedures Verify media sterilization Procure new starter cultures if needed
Nutrients contaminated	Examine microscopically Cold sterilize by 0.2 µm filtration Prepare new nutrients
Temperature too high or fluctuating	Decrease thermostat setting Remove heat sources (e.g. chillers) Install more powerful air conditioners Substitute natural illumination for some of the artificial lighting
pH too high or low	Check CO_2 supply Check regulator Increase/decrease injection frequency or injection pressure
Nutrients inadequate	Verify prescribed formula is being used Modify formula if needed
Airborne contamination	Cover culture containers Remove contamination sources (e.g. unwashed culture vessels; uncovered wastebasket; used airline)

TR 89

Microalgae Culture Protocol



Aquaculture Division

Harbor Branch Oceanographic Institution Inc.

MICROALGAE CULTURE PROTOCOL

Aquaculture Division

HARBOR BRANCH OCEANOGRAPHIC INSTITUTION INC.

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Key words: Sterile techniques, microalgae, nutrients, growth.

Abstract

The protocol for culturing microalgae at the Aquaculture Division of Harbor Branch Oceanographic Institution is explained in detail. The purpose of this document is to serve as a step-by-step guide for technicians in the microalgae laboratory. Sterile techniques, culture vessel cleaning and preparation, nutrient and media preparation, inoculations, and culture room maintenance are described. Systems diagrams and flow charts indicating culture techniques and paths are included. Appendices on autoclaving, filtration and chemical reagents preparation are also included.

Table of Contents

Introduction

At the Aquaculture Division only few species of microalgae are used to feed our organisms. The most important is the small flagellate *Isochrysis galbana* which is used to feed larval as well as juvenile and adult clams. The second most important species in our lab is *Chaetoceros gracilis*, a small diatom used mostly to feed juvenile and adult stages of clams and larval stages of shrimp and other invertebrates. Also used for the same purpose is the diatom *Thalassiosira weissflogii*. In addition to these species, the lab cultures *Ellipsodon sp.*, *Tetraselmis sp.*, *Nannocloropsis sp.*, and *Chlorella sp.*, to feed clams, rotifers, artemia and ornamental larval shrimp. Other clones not mentioned here are maintained in our small collection but they are rarely used for feedings.

Of utmost importance to the operation of growing microalgae in our laboratory is the proper utilization of *Sterile Techniques* in every step of the process in the first stages of batch culture (inside laboratory, rooms #1 and #2). Without care taken in handling containers, media and instruments, you are likely to get contamination soon after you order new stock cultures. Those cultures are not axenic[1] to begin with (axenic cultures are a myth, really!), but they are pretty clean, and will last for years without a problem if precautions are taken. Some people prefer to move cultures fast through the laboratory to avoid "crashes"[2]. I prefer to move slower, let things get dense enough, and be very meticulous with the technique to avoid those crashes. That way you are never in a rush and you do not have to live stressed and on "the edge" all the time.

Our operation starts with 10 ml sock cultures that are progressively scaled up to about 3000 liters. This guide takes you from the very beginning of the process to the stage where algae is ready to be fed to the animals in our facility.

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[1] Cultures with only one species of any microorganism. If a culture has bacteria, but still survives well, it is called "monoalgal culture".

[2] Term used when a culture abruptly dies due to contamination or nutrient depletion. Generally a crash involves decoloration of the culture, the presence of clumps and sometimes a foul smell.

1.0 Stock Cultures (10 ml)

Stock cultures are those used to start new batches of cultured algae for the laboratory. They are obtained by two different means. One is the use of a technique called Single Cell Isolation from water samples collected in the field. This technique is time consuming, difficult to master and the results are unreliable, therefore, it is not been used at Harbor Branch. The best way to obtain good quality stock cultures is to buy them from other laboratories. We use two main sources: The University of Texas (UTEX), and Bigelow Laboratories (CCMP). The collection at UTEX is extensive and the clones obtained from them in the last years have been very reliable. They are also inexpensive ($10 per test tube). CCMP also has a good collection but the cultures are considerably more expensive ($40-$100 per test tube). Cultures can also be obtained from the American Type Culture Collection (see addresses below).

1.1 Clones Used at HBOI Aquaculture

The clones used at HBOI can be ordered by letter, phone, fax, or using the internet at:

Culture Collection of Algae (UTEX)
Department of Botany
The University of Texas at Austin
Austin, Tx 78713-7460
Tel: (512) 471-4019
Fax: (512) 471-3878
URL: http://www.botany.utexas.edu/infores/utex/

National Center for Culture of Marine Phytoplankton (CCMP)
Bigelow Laboratory for Ocean Sciences
West Boothbay Harbor, Maine 04575
Tel: (207) 633-9600
Fax: (207) 633-9641

American Type Culture Collection
12301 Parkland Drive
Rockville, Maryland 20852-1776
Tel: (800) 638-6597
Fax: (301) 231-5826

1.1.1 Tiso (*Isochrysis galbana*)

We have one clone of Tiso from the UTEX collection. Its code is: LB2307

Size:	4-7 µm x 4 µm
Doublings day^{-1}:	2.89
T° Range:	16-34°C (28°C optimum)

1.1.2 Cg (*Chaetoceros gracilis*)

We have one clone of Cg from the UTEX collection. Its code is: LB2375

Size:	5-7 µm x 4 µm
Doublings day^{-1}:	4.3
T° Range:	Not determined (28-32°C optimum)

1.1.3 Tw (*Thalassiosira weissflogii*)

We have one clone of Tw also, called 'Actin,' from The University of Miami. This clone can also be obtained from CCMP.

Size:	12-24 µm diameter
Doublings day^{-1}:	1.39
T° Range:	10-30°C (25-30°C optimum)

When cultures are ordered make the people at Receiving aware of the shipment so they do not put them in the freezer or leave them exposed to the sun. Once in the lab, place them in a rack on the culture table and wait a day or two before you transfer them to new test tubes.

1.2 Culture Vessel Preparation

Stock cultures come from the providers in glass or plastic test tubes (10 ml). They are transferred into borosilicate test tubes with lose caps or lids. Tubes must be washed even if they are just out of the box. To wash tubes follow the next steps:

- submerge them in a bath of water with Alconox® detergent and leave them soaking for 24 hrs.

- Rinse them very well with Reverse Osmosis (R.O.) water several times. The last rinse is done using distilled water or deionized water. It is very important that all traces of soap are removed from inside the tubes.

- Pour 10 or 15 ml of D.I. or distilled water in each tube. This is done to trap copper residues left by the autoclaving process (The autoclave has copper plumbing) that can eventually kill the microalgae.

- Autoclave tubes for 30 minutes at 125°C (see the "Autoclaving" appendix).

- Let tubes cool down for 24 hr and store them in a dark, cool clean place until use.

1.3 Media Preparation

We buy nutrient stocks ("algae food" or f/2) rather than prepare them from chemical reagents in our lab, mainly to save time. They are bought in 5 gallon containers and re-bottled in smaller teflon or plastic containers for (internal) indoor use. We order nutrients from:

Aquacenter
Tel: (800) 748-8921
Fax: (601) 378-2862/378-2861

There are two solutions to buy:

Solution A (trace metals / catalog # 10810A)
Solution B (P, N and vitamins / catalog # 10810B)

To sterilize media for the stock cultures, seawater must be passed through a series of filters ranging from 25 µm to 3 µm, then through U.V., and finally through a fine 0.2 µm filter (Nalgene®, Fig 1.)

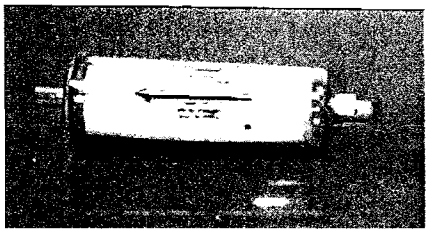


Figure 1. 0.2 µm filter

To do this make sure the filtration system is working and the U.V. lights are turned on (see the "Filtration and U.V. System" appendix). Filter water into a clean 500 ml Pyrex® glass container with a screw cap (Fig 2). Autoclave with the cap loose for 30 to 40 min. Let water cool down for 24 hr. Tighten the cap only when container is at room temperature, and store in dark, cool place until use.

Figure 2. 500 ml Pyrex® glass container with a screw cap

Nutrients are re-bottled in small (500-1000 ml) dark teflon or polypropylene containers. Do not use polyethylene bottles, they will melt in the autoclave! Apply autoclaving indicator tape (the kind with white stripes across that become black when autoclaved) to the outside of each bottle and autoclave for 30 to 40 minutes. Wash hands with alcohol before handling tubes and pipettes. Using disposable sterile Pasteur® pipettes with cotton plugs, put 1 or 2 drops of sterile nutrient solution A and solution B in 500 ml of sterile filtered seawater. **Use only one pipette per solution and container**. Discard the D.I. water that was left in each tube when autoclaved. Under the laminar flow hood (Fig. 3), and with the help of a re-pipettor or the Dispensette™ syringe (previously sterilized) distribute 10 ml of media into an empty test tube, re-cap and place it in a 36-test tube rack. Repeat the procedure until you have empty the 500 ml container.

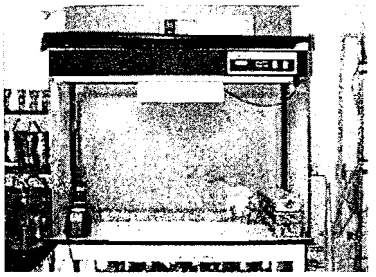


Figure 3. Laminar flow hood

500 ml can roughly fill 1.5 racks (50 tubes). Make sure you add one or two drops of silicate solution to 500 ml of sterile seawater before you distribute media to the test tubes to be used for diatoms (see the "Thiosulfate and Silicates" appendix). Usually, one rack of tubes for diatoms is prepared for each two racks of regular, non-diatom, culture tubes. Store in a dark, cool, clean place until use.

1.4 Transfers

All transfers are done under the laminar flow hood (remember to turn it on before use!). Transfers have to be done every two to three weeks so algae does not die in the tubes from nutrient depletion. To transfer, clean your hands with alcohol, get an empty test tube rack, a rack with test tubes with sterile media, and the culture rack (Fig. 4).

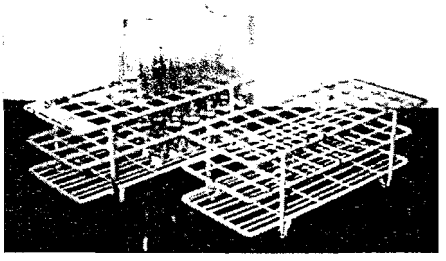


Figure 4. Culture test tube racks

Place them under the hood and using Pasteur® pipettes (previously sterilized) get 1 ml from a culture tube that has been placed momentarily in the vortex to mix it, and drop it in the new test tube. Discard the pipette (recycled pipettes are placed in a 1000 ml beaker with 10% HCl, but they can also be disposed of properly in the "glass trash" container). Repeat this operation for each tube always using a new pipette when transferring. If the pipette is dropped, or if it touches anything outside the test tubes,

discard both the pipette and the tubes to avoid any possibility of bacterial contamination. If possible, use a mask for your nose and mouth when transferring. Write the date on the record card of the cultures and in the log book, and place the newly transferred cultures and the back-up cultures on the culture table (Fig. 5).

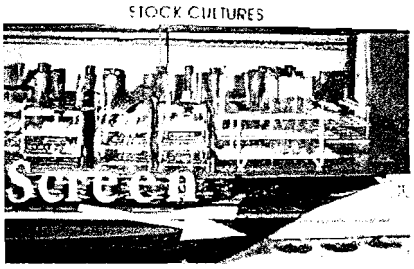


Figure 5. Stock culture table showing shade screen between lamps and cultures

1.5 Stock Culture Table

The culture table must always have two layers of screen between the lights and the cultures and the thermometer hanging in the middle should show 22-26°C. If necessary, use a small fan to cool down the pocket of air between the lights and the plexiglass. Make sure to replace any bulb that does not work or that is blinking.

2.0 Small-scale Cultures (500-6000 ml)

What we call Small-scale Cultures are those of no more than 8 liters that can be kept on the culture table. Typically we keep such cultures in clear polycarbonate containers that can be autoclaved. We have 3 sizes: 1000 ml, 4 liters and 8 liters (Fig. 6).

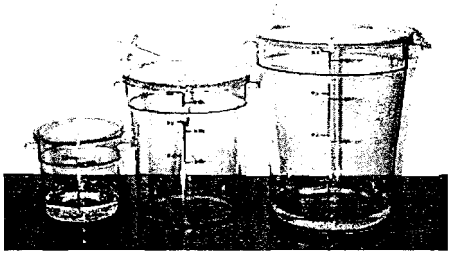


Figure 6. Clear polycarbonate culture buckets

2.1 Culture Vessel Preparation

Polycarbonate buckets have to be washed with Alconox® detergent and a sponge to remove any organic matter and bacteria from previous cultures. Even if buckets are new, they have to be thoroughly cleaned before using them to culture microalgae. After the Alconox® wash, rinse the vessel with R.O. water to remove soap and leave some water in the bucket to trap copper during the autoclaving process. Clean the lids with Alconox® soap also, and rinse them well with R.O. water. Assemble the containers put some autoclave indicator tape. Autoclave for 40 minutes.

2.2 Culture Media Preparation

Using the Filter-U.V. system, fill a 20 liter autoclavable Nalgene® container with seawater (clean it before using the same procedure you used for the buckets). Put the lid back on, but make sure to leave it loose so that the container will not explode inside the autoclave. Put autoclave indicator tape on it, and autoclave for 60 minutes. Let the water cool down for at least 24 hours to allow CO_2 levels to return to normal. Do not screw the lid back while the water is hot or otherwise the container will implode. Once it has cooled, tighten lid and store in a cool dark place (Fig. 7).

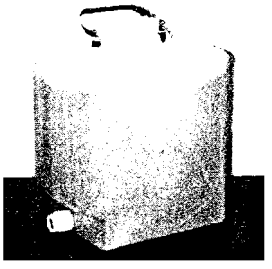


Figure 7. 20 liter Nalgene® container for media sterilization

Immediately before filling the buckets with sterile water from the Nalgene® container use an Oxford® re-pipettor with a sterile tip (they are inside the oven/incubator kept at 60°C at all times) to add 3.5 ml of each nutrient solution. Use only one tip per solution and container, and after use, place them in a beaker with 10% HCl. Later they will be washed and autoclaved for re-use. Close the container and shake to mix the nutrients. Only prepare enough media to fill all your available buckets. It is not advisable to store seawater with nutrients until the next filling because

something may grow in it, even though precautions are taken and sterile techniques are used in every step.

To fill the buckets with media take them to Culture Room # 2 and line them up on the table (clean the table with alcohol first). Clean your hands with alcohol and empty the R.O. water present in each bucket into the sink, making sure not to touch the edges of the buckets with your fingers or anything else. When you have six 4 liter containers ready (or three 8 liter containers) remove the lids and place them facing up on a clean surface. Clean your hands with alcohol again as well as the outside surface of the Nalgene® media container. Be very careful not to spill media, pour 3 liters into the bucket (you can see the mark in liters on the side) and move quickly to the next one until you finish all 6 (or all 3 if you are filling 8 liter buckets to the 6 liter mark). Put the lids back and store the buckets next to the hood in Culture Room # 1. With the left over water in the Nalgene® containers, fill the small clear buckets to the 0.5 liter mark following the same procedure.

2.3 Inoculation

There are three types of inoculations with small-scale cultures: 500 ml, 3 liters, and 6 liters. To inoculate a 500 ml clear bucket, get a back-up stock culture that looks dense, and using the vortex, mix it very well. Clean your hands with alcohol and under the hood pour the test tube culture into the bucket. Close the lid and immediately label the culture remembering to write the date. If the culture is a diatom you have to add two drops of sterile silicate solution to it. As always, use sterile pipettes for this purpose. Place the culture on the culture table until is used to inoculate the next step (Fig. 8).

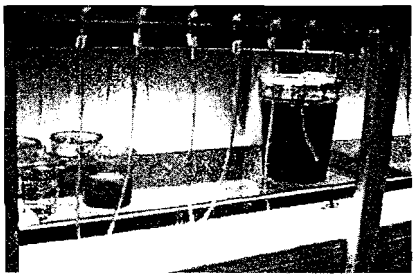


Figure 8. Small-scale culture table

4 liter buckets usually have about three liters of media. Before inoculation put a 0.2 µm filter on the air inlet. These filters are unidirectional, so make sure you install them correctly. The filters are always inside the oven/incubator at 60°C. That should be enough to maintain their sterility (Fig. 9).

Figure 9. 60°C incubator where air filters and pipette tips are kept

Using one of the 500 ml cultures, follow the same inoculation procedure (instead vortexing, just swirl the culture being careful not to spill it). If diatoms are inoculated use 10 drops of silicate solution. 8 liter buckets roughly have 6 liters of media. To inoculate them use also a 500 ml culture following the same procedure. If diatoms are inoculated add 1 ml of silicate solution. Once cultures are inoculated put them on the table and attach the air line to the filter. Turn the air on until moderate bubbling is going. Too much bubbling will probably kill a fresh inoculum, and weak bubbling will not promote good growth. Until you use them to inoculate carboys, monitor the color to see if they are getting darker.

2.4 Small-scale Culture Table

This table does not have shade screen, however, in some cases, it its good to have some screen in the section where the 500 ml cultures are sitting. Check for bad or blinking bulbs and replace them as necessary. Clean the surface of the table regularly with alcohol.

3.0 Carboy Cultures (16-20 liters)

This is the last stage at which sterile techniques can be used efficiently and cost effectively. Carboys are inexpensive, very resistant to impact and to the autoclaving process. With time they will become opaque and will brake easily, but a $6 dollar carboy can last for over a year even if is subjected to extreme temperatures and pressure once a week.

3.1 Culture Vessel Preparation

The culture containers at this stage consist of two elements: The carboy itself, and the air delivery system (I call it a "stem"). Although there are approximately equal number of carboys and stems, the latter are somewhat fragile and are broken when mishandled. Use glass for the stems because it is much more cleaner than plastic, and because it can be autoclaved. The stems consists of a polypropylene, autoclavable 250 ml beaker with a hole on the bottom, which lets through a section of thick medical grade silicon tubing (also autoclavable). At one end, a 35 cm piece of glass tubing is attached to the silicon hose. The other end is used for an air filter. Stems should be kept submerged in a chlorine bath at all times. Once they have been used in a culture they are rinsed well and placed in the chlorine bath. This bath is a Tupperware® container with a solution of R.O. water and 200 ml of 10% chlorine. Every two weeks the bath should be emptied and the container cleaned.

There are two types of carboys in our lab. The regular carboys and the "I" carboys (for Inocula) which are used to inoculate the regular carboys. There is no difference between them but they go through different processes so I have marked "I" carboys with red tape around the neck to identify them.

Carboys are cleaned as follows:

- As soon as the culture in the carboy has been used for inoculation of a larger container or other carboys, rinse it with a R.O. water "firehose" blasting the remaining algae attached to the bottom and the walls.

- Add approximately one liter of 10% muriatic acid (10% HCl) to the carboy and swirl it so the acid touches the whole surface inside. Carboys are left for a minimum of two hours with acid in them to make sure the remaining algae is killed.

- Rinse carboy with R.O. water. There are two ways to do this: One is using the firehose and the other the "serial washer" which is a 5 outlet firehose that saves a lot of time (Fig. 10) A piece of plywood with 5 holes aligned to the water jets is placed on the sink and the carboys

are turned upside down to receive the blast of water. Carboys should be rinsed for at least a minute to make sure all the acid is removed.

Figure 10. Serial washer

- "I" carboys are washed the same way but being extra careful to get rid of any possible source for contamination. Once they are clean, put one of the chlorinated stems, and a glass plug on the tip of the silicon tubing to prevent spilling during autoclaving. Also, put autoclave indicator tape.

3.2 Culture Media Preparation

Culture media preparation for the carboys follows two methods. Regular carboys are semi-sterilized with chlorine (some spores are able to survive this treatment), while "I" carboys are autoclaved to achieve total sterility.

- Put the regular carboy under the manifold that delivers the seawater (aka "the serial filler", Fig 11) and add 10 ml of 10% chlorine. Place one of the chlorinated stems in the carboy and attach the hose from the manifold to the tip of silicon tubing, remembering to open the clamp. Make sure the filters in the filter rack are not leaking and are clean. Turn the U.V. columns on and let the system run for several minutes until only seawater is coming out (see "Filtration and U.V. System" appendix). You can also fill each carboy individually.

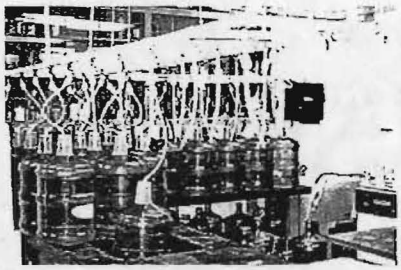


Figure 11. Serial filler

- Fill the carboys to about 16 liters and detach from the serial filler. Shake the carboy so the chlorinated water touches everything inside. It is advisable to leave the carboy chlorinating for at least 24 hr. but not for more than 6 days (Fig. 12)

Figure 12. Carboy ready to be inoculated

- "I " carboys are filled the same way but skipping the chlorine step. Instead, they are placed on the autoclave "shuttle" and taken to the big sterilizer for one hour. After sterilization, let them cool down for 24 hrs (see "Autoclaving" appendix).

To prepare the media for all carboys use sterile nutrients and an Oxford® pipette with sterile tips (Fig 13.)

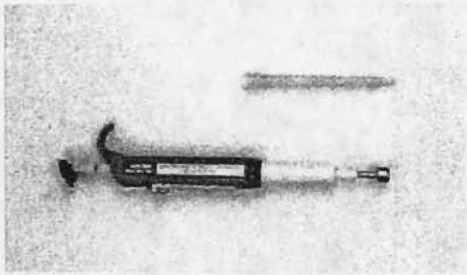


Figure 13. Oxford® pipette with sterile tip (above)

Add 3.0-3.5 ml of each solution (A & B) to the carboy being careful not to touch anything with the tip. Use one pipette tip per solution and place in a beaker with 10% HCl after use. To add nutrients, lift the top of the carboy from the upside down beaker and never touch the edges of the neck. If diatoms are going to be inoculated, add 3 ml of silicate solution to the carboy in the same fashion. Label carboys with masking tape and put a unidirectional 0.2 µm filter on the outside tip of the stem.

3.3 Inoculations

Two kinds of inoculations are performed at this stage. One is the inoculation of "I" carboys using small-scale cultures and the other the inoculation of regular carboys, using "I" carboys.

To inoculate "I" carboys, get a dense 3 or 6 liter culture and carefully pour approximately 1 liter into the carboy. Place carboy on the "Inocula" shelf in Culture Room #1 and connect the air. To inoculate a regular carboy, get one of the dense "I" carboys from the same shelf and split it into 6 regular carboys. To do this lift the stems of the carboys and balance them on one side making sure they do not fall out. In the eventual case this happens, discard that stem and replace it with a clean one. Remove the stem from the "I" carboy and with alcohol and a paper towel, clean the neck. Slowly pour 2.0-3.0 liters of culture into one regular carboy trying not to spill algae, and move to the next without returning the "I" carboy to the vertical position. This is done to prevent the culture from being spilled out of the carboy, touch the neck and then be poured back to a new carboy. Although it seems a bit extreme, this is the only way to avoid contamination. Once the 6 carboys are inoculated put them on the illuminated shelves in rooms #1 or #2 and connect the air to them.

3.4 Culture Room

Carboys are kept on shelves with fluorescent bulbs. Culture room #1 can have up to 77 carboys. I have separated the growing section from the inocula section to avoid cross-contamination and confusion. The back wall is also divided in two sections: 1) Tiso (left); 2) Diatoms (right) (Fig. 14)

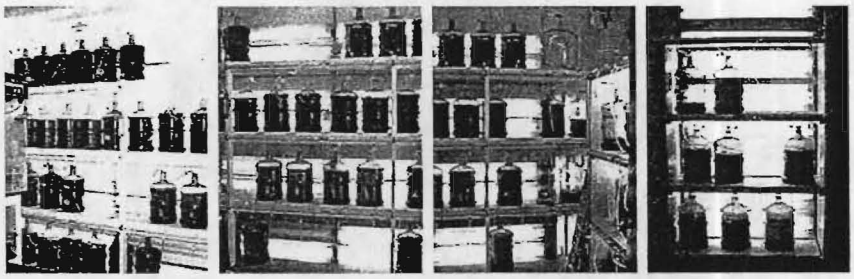


Figure 14. Carboy arrangement in culture room # 1. The picture on the left shows the Tiso section. The center pictures show the diatom section, and the left picture shows the "Inocula" section

Bulbs that are blinking or are off must be changed as soon as possible. The air system has windows of clear PVC® where you can check for fungi or moisture. If something starts growing inside the airlines they should be bleached. To bleach the airline turn the blower off, disconnect all the carboys from it and close all the valves. Pour a solution of 2% chlorine (dilute 2 parts of 10% chlorine in 8 parts of water) until you see bleach in all the windows. To make sure all air is out of the lines have a bucket at the air line drains and keep pouring bleach solution until all the air is pushed out. Leave the airline bleaching for 4 hours (it is advisable to start this procedure first thing in the morning). After bleaching, the chlorine has to be removed completely from inside the pipes. Even traces of it will kill all the cultures in a matter of minutes. To rinse the bleach you have to connect drain hoses to all the airline drains (4 in total) and connect an R.O. line to one of the inlets (top) (Fig. 15).

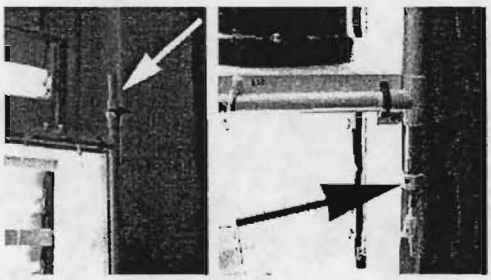


Figure 15. The left picture shows the section of pipe used to bleach the airlines. The right picture shows one of the drains at the bottom

Leave the water running for several minutes and then switch inlets and repeat the operation. Check for a change of color in the water, from yellowish to clear, and taste the water to make sure there is no chlorine in it. Turn the blower back on and open all the valves and leave it like that for three hours at least, then connect the carboys back. It would be a good

idea to connect only one carboy and wait for a few minutes to make sure nothing suddenly dies.

Provide CO_2 to the cultures to promote growth. The CO_2 tank is connected directly to a mixing chamber before the air passes through a 3 µm filter and is distributed to the carboys. I have put a flowmeter to measure the amount of carbon provided to the cultures. Usually the CO_2 flow should be set to 1000-1500 ml min^{-1}, if you need to boost the growth a bit, increase the flow to 2000 ml min^{-1} but stop there, because too much CO_2 can lower the pH of the media and kill the algae (Fig 16).

Figure 16. Air/Carbon dioxide mixing chamber

Clean the culture room every Friday at the end of the day with floor disinfectant, and wipe the work tables and hood with alcohol.

4.0 Cylinder Cultures in Room #2 (185- 208 liters)

There are two types of cylinder cultures at Harbor Branch. Clean semi-sterile cylinders in culture room #2 and non-sterile cultures in the algae greenhouse. The procedures for both are different and will be explained separately.

Culture room # 2 has 16 cylinders and space for 34 carboys. The cylinders have plexiglass lids, silicon tubing, flat bottoms and air filters. In essence, they are just big carboys and should be treated in the same fashion where the culture technique is concerned. The only difference is that they are fixed to a platform and share a common drain.

4.1 Culture Vessel Preparation

To prepare a cylinder for culture it first has to be rinsed with R.O. water using a pressure hose (garden hose connected to the R.O. line. If there are residuals from the last culture, remove them using a soft sponge and rinse again. Close the drain and harvest valves and using Alconox soap and a brush clean the inside surface of the tank. Rinse well with R.O., then using the acid sprayer (10% muriatic) spray the inside of the cylinder making sure every square cm is touched by the acid. Use a mask, safety glasses and gloves when you do this (Fig 17.)

Figure 17. Acid sprayer.

Take the lid and silicon tubing (air line) to the sink and rinse them with R.O. water, remove the airstone and put it in a container with acid. Using the sprayer's tip introduce some acid in the tubing and spray both sides of the lid. Rinse them with freshwater and replace the airstone with a dry clean one. Put the lid back on the cylinder and leave the acid for 1 hour inside the cylinder. Open the drain valve and rinse the cylinder with fresh water until all traces of acid are removed. Pay special attention when you do this because there are lights and electrical outlets close to the cylinders and it is not advisable to get them wet. Introduce the tip of the garden hose in the harvest pipe, open the valve and rinse that part of the system. Close all valves and label the cylinder with masking tape and green tape to indicate acid has been used. Leave tank as is, until you are ready to fill it with seawater (Fig. 18).

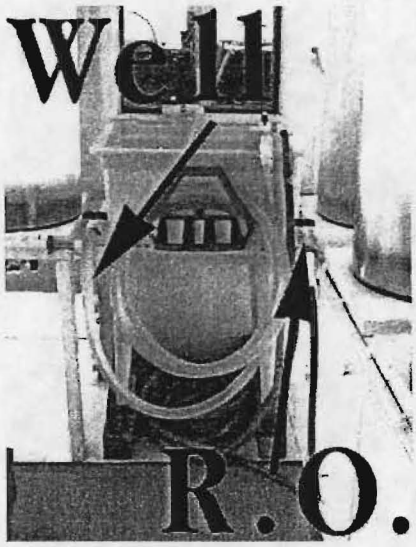


Figure 18. R.O. and well water hoses in culture room #2

4.2 Culture Media Preparation

Turn the U.V. lights on and prepare the filtration system (see appendix). Let the filtered seawater run for a while through the pipes until no more R.O. water comes out of it. Wipe the tip (shaped as an "U") of the filtered water delivery hose with alcohol and hang it on the cylinder you are going to fill. Open the valve and let the tube fill (Fig 19).

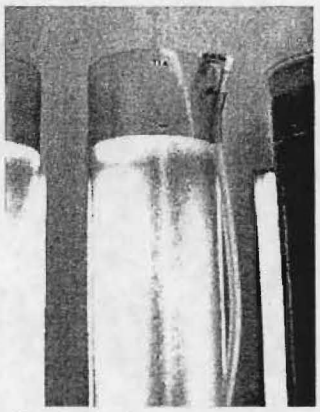


Figure 19. Cylinder in room # 2 filling

While that is going, add 100 ml of 10 % chlorine solution into the cylinder. Stop the filling when the water gets to very edge of the tube. Leave the tube chlorinating for 24 hr. Make sure the air is off. If the air is left on, the

chlorine will be blown away in a couple of hours and the cylinder will not be sterile the next day. Label the tube with yellow tape to indicate chlorine has been used.

4.3 Inoculation.

To dechlorinate a cylinder, use 15 ml of concentrated sodium thiosulfate (Fritz® Chlorine Remover, ordered from Aquacenter). Turn the air on for a few minutes to mix the thiosulfate and dechlorinate the whole volume. Check the chlorine levels with O.T.O. by taking a small sample from the harvest pipe at the bottom. This is the last place where the thiosulfate will react with the chlorine, so if you get a clean check you are ready to inoculate. If you get color, drain 500-1000 ml from the cylinder and take another sample to check. If dechlorination has not taken place at this point, you may have a bad batch of thiosulfate. Use a new bottle and repeat the procedure. Lower the level to the 200 liter mark by opening the drain valve (Fig 20).

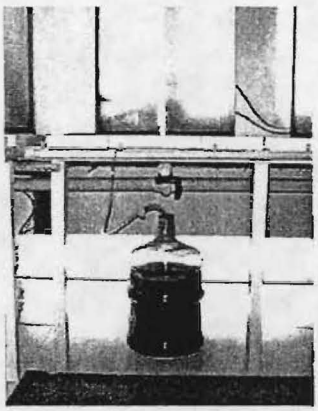


Figure 20. Inocula carboy underneath a ready to inoculate cylinder

Using a sterile 250 ml beaker, pour approximately 35 ml of sterile nutrient solution A and with a 60 cc sterile syringe get the pink liquid and add it to the cylinder. Repeat the operation with solution B (green liquid) and with silicates if you are going to inoculate a diatom. Rinse the beaker and the syringe and put them in the "to autoclave" shelve. Syringes are autoclaved inside an 8 liter clear bucket and beakers are sterilized inside biohazard bags.

Get a dense "I" carboy from underneath the cylinder you want to inoculate.

Remove the stem. Wipe the outside of the neck with alcohol. Open the cylinder by sliding the lid back a few centimeters. Slowly, trying not to splash, pour the contents of the carboy into the cylinder only touching the edge of it with the clean neck of the carboy. When you are done, slide the lid back and open the drain valve until you return the level of the water to the 200 liter mark. Do this before you turn the air back on, so only unmixed clean water is drained (the culture will be on the upper side of the cylinder). Turn the air on. Label the masking tape with red tape to indicate nutrients have been added, and write the name of the culture (clone) and the date.

4.4 Harvest.

Harvest is achieved by attaching a manifold of three outlets to the harvest drain and having 3 clean carboys at each end receiving the algae. It is best if the whole volume culture is harvested at once. Once harvesting is completed, immediately clean the cylinder before the algae dries on the walls and bottom. Clean any spills you make while harvesting. If you prefer you can harvest one carboy at the time (Fig 21).

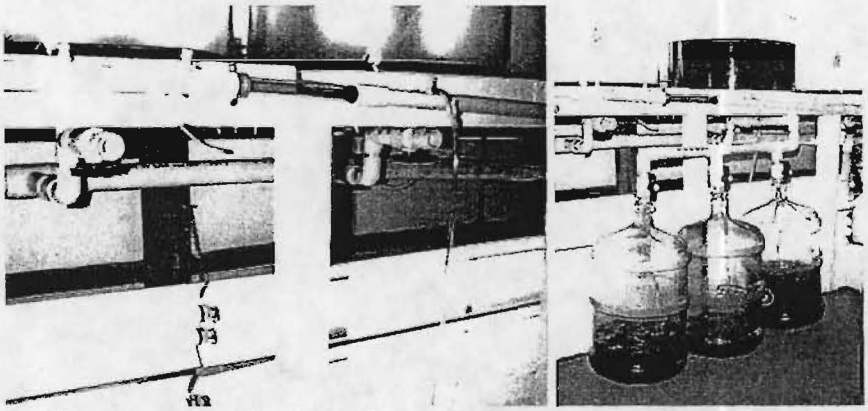


Figure 21. The picture on the left shows the common drain shared by the cylinders in room # 2. The right picture shows a cylinder being harvested to 3 carboys

4.5 Culture Room

The culture room # 2 serves a dual purpose. It houses regular cultures (cylinders) and inocula cultures (carboys). Cylinders are labeled from T1 to T16 starting on the left side of the room as you go in. Carboys are not labeled. The ambient temperature in the room should remain at 24-26°C

while the temperature inside the cultures should read 24-25°C. There is a temperature probe in cylinder T6 which has a digital display right above the tube. Before you leave the room at the end of the day check for bad bulbs and replace them if necessary. Also check that the filtered seawater and R.O. valves are closed. Water pressure can rupture a hose. If unattended, there will be an inundation in the lab. CO_2 and air systems are connected independent from culture room # 1. Check that they are running ok.

5.0 Algae Greenhouse Cultures

In this section even though there are different volume cultures, I will lump everything in one procedure since it is basically the same for all sizes. The main difference with the cultures inside is the level of cleanliness that can be achieved and maintained in the greenhouse. Since it is mainly an "outdoor" operation, cultures tend to get more contamination. Also, the temperature and light parameters are, for the most part, uncontrollable. During the winter months the water gets relatively cold for tropical species like T-iso and since days are shorter, their photosynthetic time is reduced, thus, reducing the growth. At the same time, winter bring less organisms in the water and contamination is easier to control. During the summer months, the temperature raises to intolerant levels in the greenhouses and the water is much more "diverse" in its microfauna and flora, which represents a major problem when trying to minimize contamination. Days are longer during this time and cultures grow very fast but they also crash faster. It is very difficult to control these factors because the system does not allow it. The best thing to do is to be extra careful with the technique and monitor the system often. We are subjected to a number of risks such as the many seasonal ecological successions of our water supply and its regular chemical and salinity changes. There are facts of life that some cultures are going to crash, and that it will be very difficult to pinpoint the cause. Clams WILL be affected by the quality of the algae and since we are producing more than ever, the risk of loosing seed clams due to algae problems is higher. Be prepared for this eventuality.

5.1 Culture Vessel Preparation

Cleaning for cylinders, barrels and tanks is basically the same. They are rinsed well with R.O. water, and only when Tiso is going to be inoculated acid, is used as an extra precaution. After the acid, tanks are rinsed again with fresh water. Airstones are changed with each new culture for Tiso, and every 3 culture cycles for the rest of the species (Fig. 22).

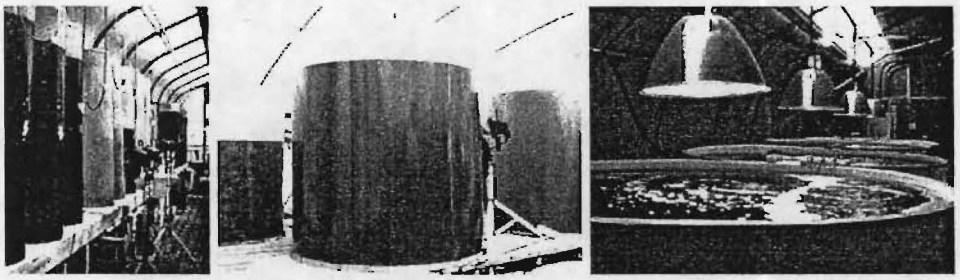


Figure 22. Cylinders, barrels and tanks where microalgae is cultured in the greenhouse. The center picture shows portable lights next to a 5' culture barrel

5.2 Culture Media Preparation

For this section I will present the protocol in the form of a table (Table 1). Vessels are filled with filtered seawater, chlorinated, dechlorinated, and nutrients are added before inoculation using clean graduated plastic cylinders.

Table 1. Media preparation procedure.

Vessel Type	Water Filter Size (µm)	Chl. (ml)	Thio. (ml)	Sol. A (ml)	Sol. B (ml)	Si (ml)
Cylinder	3	100	18	30	30	20
Barrel (4')	3 (Tiso), 5 (others)	300	100	150	150	80
Barrel (5')	3 (Tiso), 5 (others)	400	150	400	400	150
Tank (8')	5	500	200	600	600	200

The main concern while preparing the tanks and media for inoculation is to use clean instruments such as pipettes, graduated cylinders, hoses, etc., and to inoculate as soon as the media is prepared. Most culture vessels in the greenhouse do not have covers, or have very precarious lids, which contribute to, or perpetuate contamination problems. It is a priority to

daily check for fungi and other microorganisms growing in the air and water lines, and if necessary, to bleach those lines on a regular basis.

5.3 Inoculations

To inoculate greenhouse cylinders, carboys from the inside lab are used. They can be regular carboys from Culture Room #1 or carboys harvested from the cylinders in Culture Room #2. Barrels (4') are inoculated using either carboys from inside, or outside cylinders. Barrels (5') are inoculated the same way and tanks (8') are inoculated with barrels. Finally, clams are fed with algae from cylinders, barrels or tanks either by centrifuging (with a continuous centrifuge located outside the algae green house) and collecting a concentrated paste that is later selectively diluted in the clam hatchery, or by pumping the algae to head tanks that will trickle to the clams.

5.4 Greenhouses

The green houses have clear tops to allow light through but there is also artificial illumination that has to be maintained. Check regularly for bad bulbs, and during cloudy days use the portable lights to boost the growth of the cultures. CO_2 is very important at this stage and must be checked often. Again, the key to avoid contamination problems is to be on top of the situation, clean everything well, constantly check for suspicious colored spots in all the hoses, and monitor the progress of the cultures frequently. Cleaning the greenhouse on a regular basis is a must when ever time permits (Fig 23).

Figure 23. Greenhouse

Figure 24 summarizes the technique followed at the HBOI Aquaculture Division to culture microalgae.

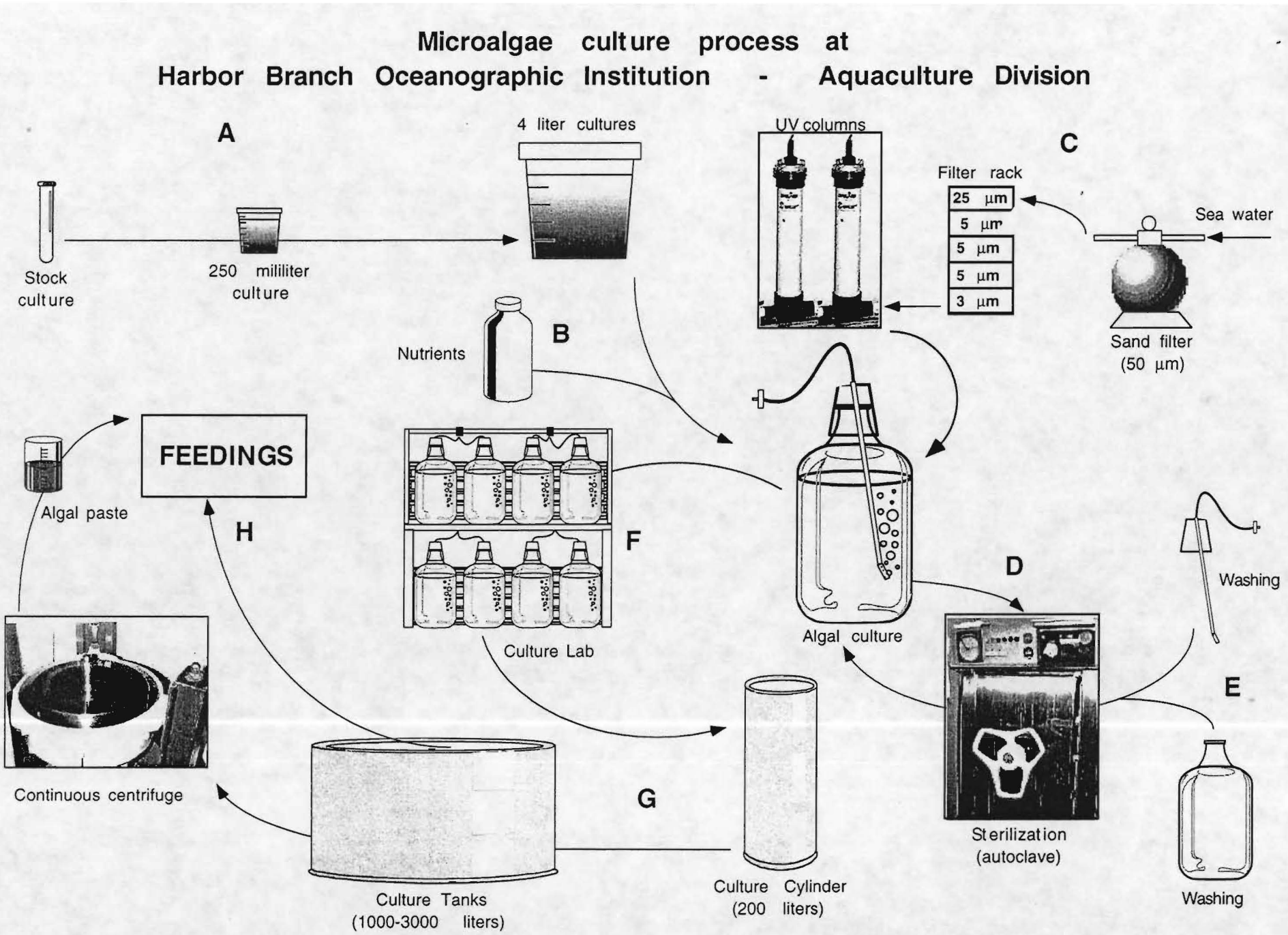


Figure 24. **Microalgae culture process** . A) Scaling up cultures; B) Nutrient preparation and addition; C) Sea water filtration and UV exposure; D) Sterilization; E) Culture vessel washing; F) Culture inoculation and mass algal cultures; G) Culture volum e expantion H) Algae used for feedings,

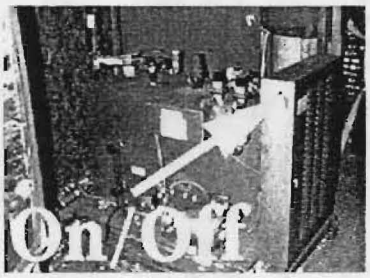
Appendix I

Autoclaving

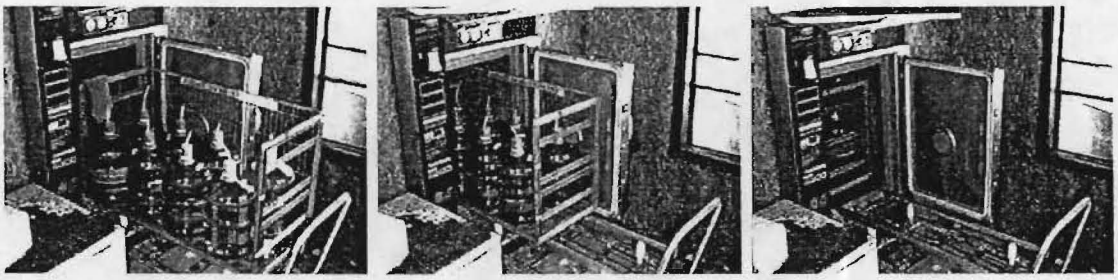
We have two autoclaves. A small one with capacity for only a few flasks, and an industrial one which can hold up to 10 carboys at once. The instructions for the operation of the small autoclave are written on the front of it. Use this autoclave when you need to sterilize small instruments such as pipettes, test tubes, small tubing, and nutrients. The big autoclave is used almost exclusively for sterilizing carboys.

To operate this autoclave follow these steps:

1- Turn the boiler on



2- Put carboys or buckets in the chamber by sliding in the top of the autoclave cart or shuttle.



3- Turn power and control switches on. You will get an error message on the display window followed by a print out. Then a beeping sound will follow for a few seconds and then the temperature of the chamber will be displayed. Some times, since the autoclave resides outside and the humidity has ruined some electronic components, the temperature will not be displayed properly.

4- Wait 30-40 minutes for the pressure in the Jacket to raise up to 30 psi. Read this off the gauge.

5- Once the jacket pressure reaches 30 psi, close the door making sure is tight, and turn the water on (blue valve). Leave the two red valves alone. Using the manual controls turn the knob to the "condition" position and wait for 1 minute until the pressure of the jacket decreases to 10 psi.

6- Turn the same knob to the "sterilize" position and time the cycle using the stopwatch. For carboys with filtered seawater or Nalgene®

20 liter containers, you should allow 60 minutes. For buckets 40 minutes will be enough.

5- After the time is up, turn the knob to "fast exhaust" and close the blue valve. Steam will rush out of the autoclave exhaust outside the room. Make sure there is nobody standing near by.

6- Wait approximately 30 to 40 minutes for the pressure of the chamber to go all the way down. The autoclave door will not open if the pressure is above 0. Nevertheless the security system may fail, so use extra care any time you try to open that door. Remember that the temperature inside is over 124°C (250°F).

7- Stay away from the steam that comes out when you open the door and wait a few minutes until the carboys are nor boiling. They have been under pressure and the temperature they supported was above the boiling point of seawater. Some times the water gets into a state called "super heated water" and even though the ambient pressure has return to 1 atmosphere, the temperature of the water remains above the boiling point. The slightest movement will suddenly make the entire carboy to boil in a few seconds. You do not want to be close if that happens. Leave the carboys cooling inside with the door cracked for at least one hour.

8 - Turn controls off and then turn the boiler off.

9- Using the orange autoclave gloves pull the cart out of the autoclave and drag it back to the lab. Have a sign warning people that the cart is hot.

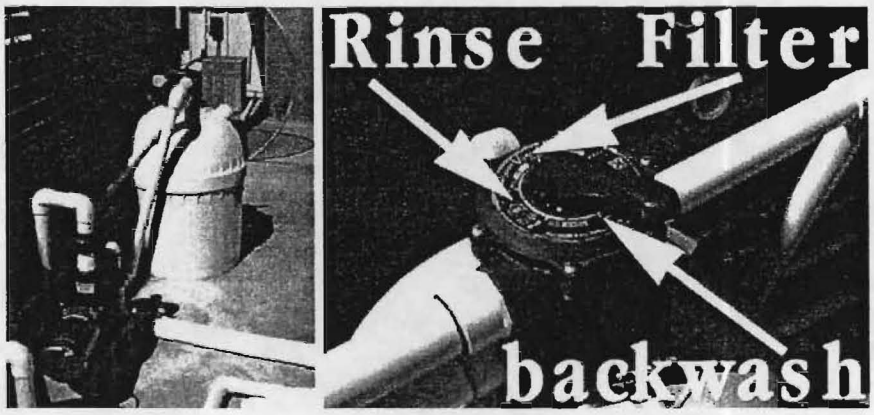
10 - If there were spills inside the chamber, wait until it is cold and rinse the inside with a hose.

If the autoclave is not working or if you notice something out of the ordinary, call AMSCO (Tel: 1800-333-8828) Tell the operator that you are referring to the HBOI Aquaculture autoclave. We have a preventive service and maintenance agreement with them.

Appendix II

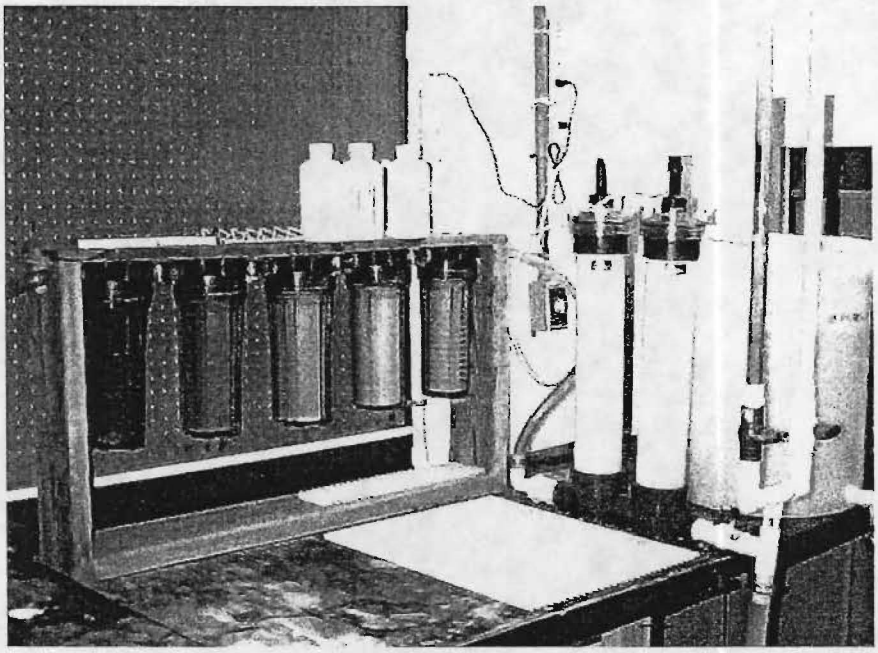
Filtration and U.V. System

There is a rudimentary filtration system in place at the laboratory. The water comes out of the well and passes through a de-gaser where a myriad of micro organisms thrive. Then the water goes to a reservoir with its own diverse ecosystem. Here also, there is an accumulation of sediment and organic matter which provide surface area for bacteria to grow very nicely. The water is then pumped through a sand filter and then it goes to the laboratory where a series of 5 filters and a U.V. dual column are installed to try to stop those organisms from going into our cultures. After the water goes through these filters, it is distributed to the carboys or cylinders where it is chlorinated or autoclaved. The sand filter has to be back flushed every time you want to use filtered water. To back flush turn the lever to the " back flush" position and wait for 5 minutes until the water coming out of the drain looks clear. Turn the lever to the "rinse" position for one minute and finally turn the lever to the "filter" position. Inside the lab, prepare the filter rack by putting a set of clean filters.

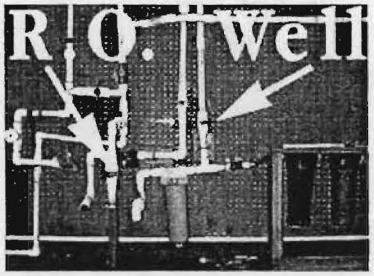


When you are ready to use the water turn the U.V. system on and open the well water valve and the by-pass valve but keep the filter rack valve closed. This way the pipes can be purged without clogging the filters. Once the water comes out clean, close the by-pass valve and open the filter rack

valve. Usually these filters are sitting in chlorinated R.O. Water so you have to let the well water run for a few minutes. You can use this water in three ways: 1) Use the extension hose to fill carboys that are not sitting under the serial filler, to fill Nalgene® containers for the preparation of media, or carboys in the autoclave shuttle. 2) Use the serial filler to fill 45 carboys at once. 3) Use the extension hose in culture room #2 to fill the cylinders.



After any of these uses, you have to back flush the pipes with R.O. water so nothing grows in them until the next filling. For that purpose, open all the valves in the system and connect the R.O. hose to the extension hose you used. Open the by-pass valve and turn the R.O. water on. Wait a few minutes until only fresh water is coming out. You can then pour 60 ml of chlorine inside the R.O. Line and turn it on again. This will take high concentrations of chlorine to the filters. As soon as you smell the chlorine coming out of the by-pass pipe turn to R.O. water off and close all the valves.



Clean the filters with Micro® every other filling or alternate the filters each filling with a new or clean set. One set of filters should no be used more than 10 times

Appendix III

Thiosulfate and Silicates

We use two kinds of thiosulfate solutions. One we do not have to prepare because we buy it from Aquacenter. It is called "Chlorine Remover" and it is very concentrated. We use chlorine remover for the dechlorination of carboys and cylinders. The other type of thiosulfate we prepare from an industrial powder and it is not as concentrated. We use it for barrels and tanks. To prepare this solution dissolve 250 gm of the powder into 1 liter of water on a magnetic stir plate.

Silicates are used in the diatom cultures because diatoms build a hard silicate shell around their cell wall. The silicate solution is prepared dissolving 200 gm of sodium metasilicate in one liter of water. You will need a magnetic stirrer and a hot plate to help the dilution processes.

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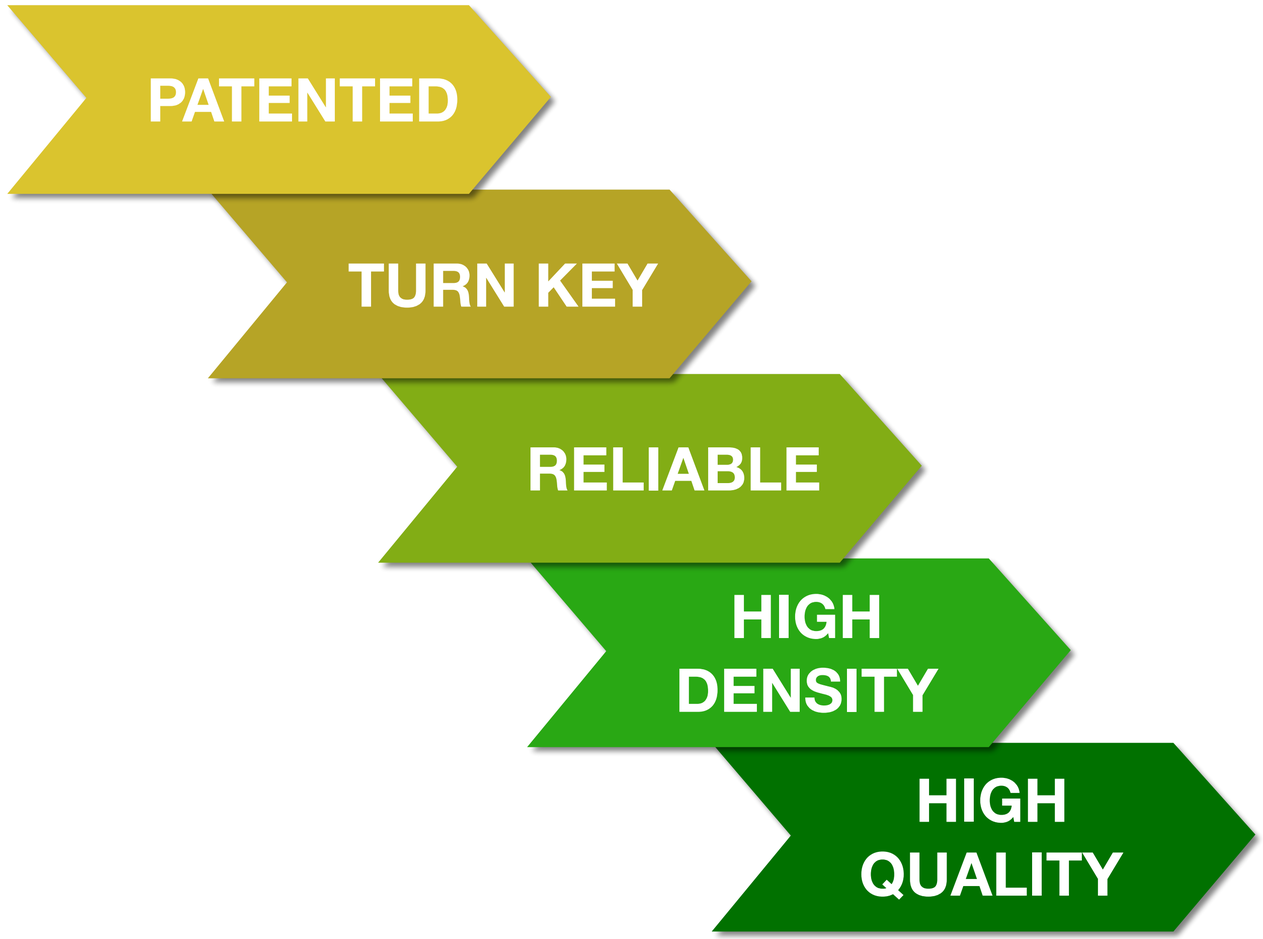

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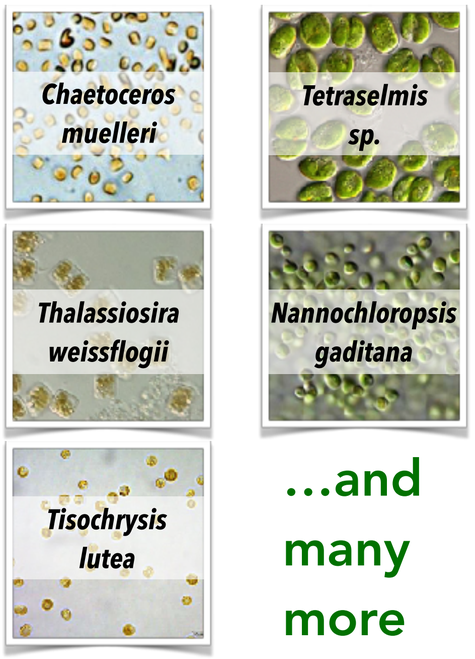

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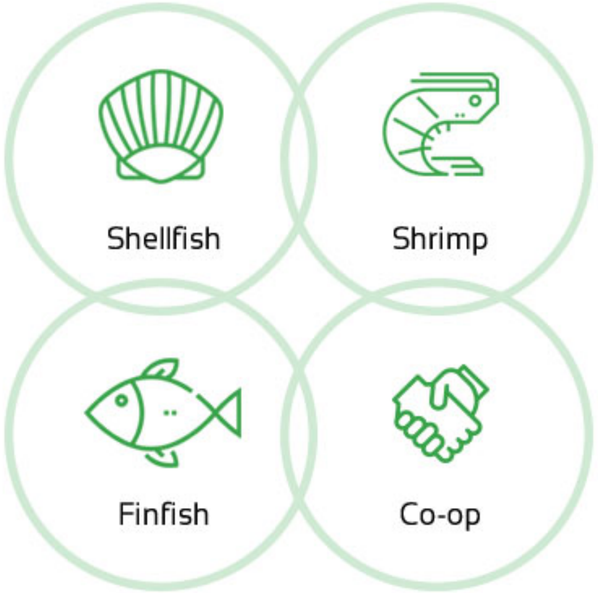

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Higher profits

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The Algal Revolution

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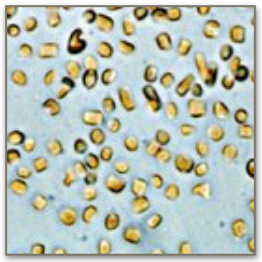
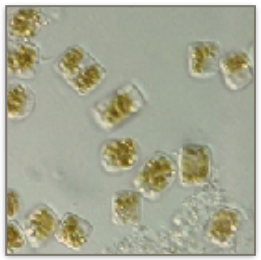
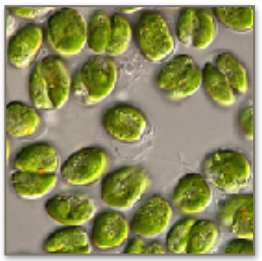
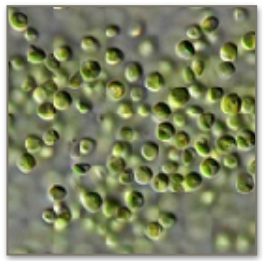
	Tisochrysis lutea	*Chaetoceros muelleri*	*Thalassiosira weissflogii*	*Tetraselmis sp.*	*Nannochloropsis sp.*
					
Density range (million cell/mL)	30-40	40-50	5-6	10-12	100-300
Size range (micron)	5-10	5-20	15-30	10-20	3-5

AlgaFeed has broken the barrier to produce high density microalgae on a commercial scale

We provide the complete range of live, natural, high density algal strains delivered to the door as a service

Allow leading shrimp, shellfish and fin-fish hatcheries to substitute sub-optimal in-house grown microalgae with AlgaFeed's portfolio of high density microalgae of unequaled nutritional value

- Reliable supply
- Consistent quality
- Increased survival
- Faster growth
- Higher yields
- Better quality seed
- Higher profits