

## MARINE FISH PRODUCTION MANUAL

### CHAPTER 1: ALGAE

#### STOCK TUBE CULTURE

The algae cultures maintained in tubes are the backbone of the production facility. These cultures are subcultured (tube to tube) every three weeks to indefinitely maintain a low-level growth of contaminant-free (parent) stock, with a minimal amount of effort. Subcultures from tube to flask are made only in emergency situations, such as when the density of the flask culture crashes or the culture becomes heavily contaminated due to improper aseptic transfer techniques.

Three replicates of each species or strain of algae are subcultured (tubes A-C in Figure 4). Tubes A and B are kept in the stock room, whereas tube C is kept in the mass culture room to assure against total loss of the culture due to an electrical failure or other catastrophe in the stock culture room. Subcultures are made from tube A only; the other two replicates remain unopened. As an extra precaution, the previous two subcultures are also retained so that problems can be back traced. Thus there are 9 tubes for each species of algae (see Figure 4).

#### Glassware Preparation

1. Acid wash and rinse tubes as previously described.
2. Fill tubes with 10-15 ml of the appropriate Tube/Flask Enriched Seawater Media and screw caps on loosely. Label or distinguish tubes containing silicates from those without silicates.
3. Autoclave. Allow to cool before inoculation.

#### Tube Transfer

1. Add 0.01 ml of autoclaved vitamins to each tube using a sterile cotton plugged serological pipet (or add 1 drop from a Pasteur pipet) following the strict aseptic techniques previously described. It is most efficient to add vitamins to all tubes first.
2. Shake tube A (three week old parent culture) to resuspend any cells on the bottom and withdraw  $\frac{1}{2}$  of a Pasteur pipet full of culture. Add 4-5 drops of algae into each of the three replicate tubes. Cap loosely. Save algae remaining in pipet for microscopic examination. Label and number tubes (see below).
3. Place all A-C Tubes in a rack on the shelf in the stock room in direct light for 1 week to establish growth. After 1 week, transfer all tubes to an indirect light source, integrating the tubes in the proper order and discarding the oldest set of cultures (see Figure 4).

## Culture Evaluation

Using the hemacytometer, examine the culture sample remaining in the transfer pipet to check for live cells, density and evidence of contamination. Record comments on the Stock Tube Evaluation Sheet (Table 6).

## Numbering Tubes

Record on each tube the species, date and replicate of the parent tube from which the transfer was made, and the date and replicate of the present transfer. See Figure 4 as an example.

## Resuspension

Resuspend (shake) the tube cultures once a week. If a Vortex is not available, the next best method is accomplished by holding the tube and cap between the thumb and forefinger of one hand and tapping the bottom of the tube vigorously with the forefinger of the other hand. This creates a vigorous circular motion within the tube that resuspends the culture effectively. Generally, Tetraselmis will stay in suspension while Chaetoceros settles to the bottom. A culture that “wisps” into suspension indicates that it had settled unclumped.

## FLASK CULTURE

The flask cultures represent the first step in the sequence of working algae cultures. These cultures are maintained in the stock room and new cultures are started from them twice a week. Flask cultures take 3-4 days to reach a density that can be inoculated into a carboy. The following describes a schedule that can be used to culture and transfer Chaetoceros and Tetraselmis in flasks.

### Glassware Preparation (Monday and Thursday)

1. Gather enough flasks (125 ml) to subculture 4 flasks per species. The number of flasks needed can be increased depending on the number of carboys that will be inoculated per week. As a rule, subculture 8 flasks/week to inoculate 2 carboys/week; or 12 flasks/week for 3-4 carboys/week. Acid wash and rinse as previously described.
2. Fill flasks with 50 ml of the appropriate Tube/Flask Enriched Seawater Media. Cap with heavy-duty foil. Autoclave. Cool. Label flasks.
3. Check to make sure that there are sufficient number of sterile pipets and quantity of vitamins for the transfer. These can be autoclaved with the flasks if necessary.

## Flask Transfer (Tuesday and Friday)

1. Add 0.05 ml of autoclaved vitamins to each flask using a sterile serological pipet (or add 2-3 drops from a Pasteur pipet) following strict aseptic techniques previously described. It is most efficient to add vitamins to all flasks first.
2. Resuspend (swirl) the culture in the parent flask. Withdraw a full Pasteur pipet from the flask making sure not to wet the cotton plug. Add 1/4 of the pipet (approx. 1ml) of algae to each of the 4 flasks. Recap with foil. Save the algae remaining in the pipet for microscopic examination. Label and number flasks (see below).
3. Place flasks in direct light in the stock culture room. Discard the oldest cultures. Swirl flasks daily to resuspend settled cultures.

## Culture Evaluation

Using a hemacytometer, examine the culture remaining in the transfer pipet to check for live cells and contamination. Look for normal activity, color, size, shape, density, etc. Record observations on the Flask Culture Evaluation Sheet (Table7).

## Numbering Flasks

Record on each flask the species, the date and replicate of the parent flask from which the sample was taken, and the date and replicate of the present transfer. A star "\*" on the flask will identify which flask has been used for the transfer. See Figure 5.

## CARBOY CULTURE

Carboy cultures represent the second step in the sequential culture of algae. Carboys are 9 or 12 liters in capacity. All cultures are aerated to keep cells in suspension and in contact with the light. Each carboy is supplied with an aeration apparatus consisting of a rubber stopper, inlet glass tube that extends to near the bottom, and a short glass tube for air escape. Carboys are inoculated twice a week (more often if algae demand is high) from flasks. Normally it takes 4-5 for a flask culture to reach a density that can be inoculated into the next step, mass culture. The following describes two schedules which can be used for carboy inoculations with Chaetoceros or Tetraselmis.

## PREFERRED METHOD

### Glassware Preparation (Tuesday and Friday)

1. Acid wash and rinse, with DI water and seawater, one carboy per species. Fill carboy with seawater from the reservoir (See Figure 6). Insert aeration apparatus, but do not aerate.
2. Add clorox (sodium hypochlorite 5.25%) at a rate of 0.08 ml/liter of seawater to disinfect. Aerate a few seconds to distribute clorox. Let stand 30-45 minutes.
3. Neutralize clorox with sodium thiosulfate at a rate of 0.22 ml of stock solution/liter of seawater. (Prepare sodium thiosulfate stock solution by dissolving 2 g of sodium thiosulfate in 200 ml of DI water.) Aerate for 30-45 minutes. Carboy is now ready for inoculation.

### Carboy Inoculation (Tuesday and Friday)

1. Enrich seawater with Secondary Stock Nutrients at a rate of 1 ml nutrient/liter of seawater.
2. Inoculate with 100 ml (2 flask cultures of algae).
3. Label and date carboy.

## MASS CULTURE TANKS

The mass culture tanks (300 liter capacity) are the final step in the sequential culture method. It is from these tanks that algae is taken to feed the shrimp in the larval rearing tanks. The mass culture tanks are maintained under constant light and temperature conditions in the mass culture room. All tanks are aerated (See Figure 7) such that a circulation is created which exposes all algae cells to illumination for photosynthesis.

Tetraselmis takes about 6-8 days to reach a density of  $1 \times 10^6$  cells/ml, although this species is usually fed at a density of  $5-8 \times 10^5$  cells/ml. Chaetoceros takes 3-5 days to reach  $2 \times 10^6$  cells/ml and is usually fed at a density of  $1-3 \times 10^6$  cells/ml. Shrimp are always fed algae that is still in log phase (exponential) growth because the algae is more nutritious during the period of rapid growth than when in the stationary phase of growth.

### Tank Preparation

1. Empty tank by bucketing or pumping dry. The submersible pump must be cleaned with hot water after each use to prevent contamination in other tanks. To clean, fill the half sink with hot water and pump through the body and tubing. Disassemble airlines; discard air stones (they crumble in hot water). Air lines can be reused after soaking in

hot water. Scrub tank with Alconox and clorox. Rinse well with hot water. Set on side to drain. Return to position in mass culture room.

2. Fill tank with reservoir seawater. Reassemble air lines with new air stones. Do not aerate. Disinfect with clorox at a rate of 0.04 ml/l seawater. Aerate a few seconds to distribute clorox completely. Let stand 30-45 minutes.
3. Neutralize clorox with sodium thiosulfate at a rate of 0.11 ml of stock solution/1 seawater (see section on carboy preparation for preparation of stock sodium thiosulfate). aerate for 30-45 minutes. Tank is now ready for inoculation.

NOTE: 0.04 ml/l translates to 4 ml clorox/100 l and 0.11 g thiosulfate/100 l  
0.08 ml/l translates to 8 ml clorox/100 l and 0.22 g thiosulfate/100 l

#### Tank Inoculation

1. Aerate and enrich seawater with Tertiary Stock Nutrients at a rate of 1 ml nutrient solution/6 liters of seawater (F/4 strength in final concentration).
2. Inoculate with the contents of one 12 l carboy culture which is 4-5 days old. Or inoculate with 10 l of a mass cultured Chaetoceros culture which has a density of  $1.5-2.0 \times 10^6$  cells/ml and culture will reach feeding densities in 2-3 days. Or inoculate with 12 l from a mass cultured Tetraselmis culture which has a density of  $4.0-5.0 \times 10^5$  cells/ml and the culture will reach feeding densities in 3-4 days. A tank which is in healthy condition, but has half the volume at the end of the day after feeding can be brought back up to full volume by adding reservoir seawater directly to the tank without disinfecting with clorox; enrich the volume added with the appropriate quantity of Tertiary Stock Nutrients at an F/4 strength.
3. Diatom cultures can be re-enriched with Tertiary Silicates every 2-3 days (use F/4 strength) if necessary.

Cultures should be examined on a daily basis beginning day 3. Record observations on density, contamination level, cell size and shape on the Algae Production Data Sheet (Table 3).

## ENRICHED SEAWATER MEDIA AND NUTRIENT STOCK SOLUTIONS

To facilitate algal growth, natural seawater is “enriched” with various inorganic nutrients, most notable nitrates and phosphates. Certain species also require vitamins, trace metals and in the case of diatoms, silicates. McLachlan (1973) lists several types of enriched seawater media preparations. The medium used by Texas A&M is Guillard’s F/2 (Guillard, 1975).

Guillard’s medium calls for the preparation of four separate nutrient solutions: nitrates and phosphates, silicates, vitamins and an iron/EDTA/trace metal combination. Because of the low concentration of the vitamins and trace metals needed, these solutions are prepared as Primary Stock Solutions which are diluted to prepare the Working Stock Solutions. For convenience, two sets of Working Stock Solutions are kept, one for tube, flask and carboy enrichments (Secondary Nutrient Stock solutions) and the other for mass culture enrichments (Tertiary Nutrient Stock Solutions). The difference between the two sets is merely a concentration factor which gives a convenient volume to add to each size of culture vessel. The compounds required to prepare these nutrient solutions are listed in Table 4 and should be referred to when reading the preparation guidelines that follow. Table 5 lists the chemical formulas for these compounds, plus purchasing information.

### PRIMARY NUTRIENT STOCK SOLUTIONS

#### Preparation of Primary Vitamin Stock Solution

The rationale behind the mixing instructions for the Vitamin Stock Solution is presented below. This rationale serves as a model for the mixing instructions of other nutrient solutions.

#### I. Vitamin B<sub>12</sub>

##### A. Facts:

1. B<sub>12</sub> is packaged in a bottle containing 0.1 g = 100 mg = 100,000 ug.
2. Guillard’s F/2 medium required 150 ug of B<sub>12</sub>/tank.
3. Thus, a 300 liter mass culture tank requires 150 ug of B<sub>12</sub>.
4. The stock jug for holding the vitamin solution has a volume of 4000 ml.

##### B. Therefore:

1. To enrich a tank with 150 ug of B<sub>12</sub>, in a stock volume of 100 ml (for convenience), the 4000 ml stock jug will contain 40 tanks worth (300 l of seawater/tank) of B<sub>12</sub>.
2. In other words, a 4000 ml stock jug would contain a total of 6000 ug of B<sub>12</sub>, a quantity sufficient to enrich 12,000 liters of seawater media at 0.5 ug/l (0.33 ml of vitamin stock/1 of seawater media).

#### II. Biotin

##### A. Facts:

1. Biotin is also packaged in a bottle containing  $0.1 \text{ g} = 100 \text{ mg} = 100,000 \text{ ug}$ .
2. Guillard's F/2 medium requires  $0.5 \text{ ug}$  of Biotin/liter of seawater.

B. Therefore:

1. All calculations for Biotin would be the same as for  $B_{12}$ .

### III. Vitamin $B_1$

A. Facts:

1.  $B_1$  is packaged in a bulk bottle in powdered form.
2. Guillard's F/2 medium requires  $0.1 \text{ mg}$  of  $B_1$ /liter of seawater.
3. The mass culture tanks hold 300 liters of seawater and thus require an enrichment of  $30 \text{ mg}$  of  $B_1$ /tank.
4. The stock jug has a volume of 4000 ml.

B. Therefore:

1. To mix all three vitamins together, a 100 ml dose of Vitamin Stock must contain  $30 \text{ mg}$  of  $B_1$ , as well as  $150 \text{ ug}$  of  $B_{12}$  and  $150 \text{ ug}$  of Biotin.
2. A 4000 ml stock jug will supply 40 100 ml enrichment doses and therefore must contain a total of  $12000 \text{ mg}$  ( $1.2 \text{ g}$ ) of  $B_1$ .

### IV. Preparation of a Primary Vitamin Stock Solution Containing $B_{12}$ , Biotin and $B_1$ .

A. Facts:

1.  $B_{12}$  and biotin are packaged in quantities of  $0.1 \text{ g} = 100 \text{ mg} = 100,000 \text{ ug}$  each.
2.  $\frac{100,000 \text{ ug}}{6000 \text{ ug}/41 \text{ jug}} = 16.67 \text{ jugs}$  worth of  $B_{12}$  and biotin
3.  $(1.2 \text{ g of } B_1/41 \text{ jug}) \times (16.67 \text{ jugs}) = 20 \text{ g of } B_1$ . In other words,  $20 \text{ g}$  of  $B_1$  are needed to equal the amount of  $B_{12}$  plus Biotin as packaged.

B. Mixing Instructions:

1. Dissolve  $20 \text{ g}$  of  $B_1$ ,  $0.1 \text{ g}$   $B_{12}$  and  $0.1 \text{ g}$  Biotin in 500 ml DI water. Measure a second 500 ml quantity of DI water and use part of it to rinse the residual vitamin powder from the small bottles. Adjust final volume to 1000 ml.
2. When thoroughly mixed:  
 $\frac{1000 \text{ ml}}{16.67 \text{ jugs}} = 60 \text{ ml of Vitamin Stock}/4 \text{ liter jug}$

3. Measure 60 ml of Vitamin Stock solution into each of 16 plastic bottles for freezing. Date and label each bottle as:  
**“Primary Vitamin Stock Solution containing 6000 ug of B<sub>12</sub>, 6000 ug of Biotin and 1.2 g of B<sub>1</sub>. Add to 4000 ml of DI water and use at 1 ml solution/3 liters seawater for F/2 strength.”**  
This frozen Primary Stock solution will be used later to prepare the Tertiary Vitamin Stock Solution (refer to Table 4).
4. The remaining primary stock solution (approx. 40 ml) is sufficient to prepare 2667 ml of Secondary Vitamin Solution and should either be used immediately or frozen in 2-20 ml portions, labeled in such a manner so as to distinguish them from the 60 ml portions.
5. Refrigerated portions of Primary Vitamin Solution should be used immediately. Frozen portions can be stored indefinitely, although a six month supply is recommended as a maximum.

#### Preparation of Primary Trace Metal Stock Solutions

Each metal (copper, zinc, cobalt, manganese and molybdate) is prepared as a separate stock solution. Table 4 lists the amount of each metal needed to prepare a solution. Copper is used as an example.

1. Dissolve 9.8 g of cupric sulfate in 1 liter of DI water. Refrigerate in a brown plastic bottle.
2. Date and label as: **“Primary Trace Metal Solution for Copper containing 9.8 g of cupric sulfate/liter.”**

The Primary Trace Metal Stock Solutions will be used later to prepare both the Secondary and the Tertiary Trace Metal Stock Solutions.

#### SECONDARY NUTRIENT STOCK SOLUTIONS

The Secondary Nutrient Stock Solutions are prepared for tube, flask and carboy seawater enrichments. The Nitrate/Phosphate and Silicate Secondary Stock Solutions are prepared from reagent grade chemicals. The Vitamin and Trace Metal Secondary Stock Solutions are prepared from the Primary Stock Solutions (Table 4). For an F/2 strength enrichment, use 1 ml of each nutrient/1 of seawater.

#### Preparation of Secondary Nitrate/Phosphate Stock Solution

1. Dissolve 300 g of sodium nitrate and 20 g of sodium phosphate in 4 liters of DI water. Refrigerate in a Nalgene container.
2. Date and label as: **“Secondary Nitrate/Phosphate Stock Solution for Tubes, Flasks and Carboys. Use 1 ml nutrient solution/liter of seawater.”**

#### Preparation of Secondary Silicate Stock Solution

1. Dissolve 120 g of Sodium Silicate in 4 liters DI water. Refrigerate in a Nalgene container.
2. Date and label as: **“Secondary Silicate Stock Solution for Tubes, Flasks and Carboys. Use 1 ml nutrient solution/liter of seawater.”**

#### Preparation of Secondary Vitamin Stock Solution

1. Defrost one 20 ml frozen portion of Primary Vitamin Stock Solution. Mix with 3.980 liters DI water. Refrigerate in a Nalgene container.
2. Date and label as: **“Secondary Vitamin Stock Solution for Tubes, Flasks and Carboys. Use 1 ml nutrient solution/liter of seawater.”**

#### Preparation of Secondary Trace Metal Solution

1. Dissolve 12.6 g of ferric chloride, 17.4 g of EDTA and 4 ml of each of the five Primary Trace Metal Solutions in 4 liters DI water. Refrigerate in a Nalgene container.
2. Date and label as: **Secondary Trace Metal Solution for Tube, Flask and Carboys. Use 1 ml nutrient solution/liter of seawater.”**

Alternatively, the Secondary Stock Solutions can be prepared by diluting the Tertiary Stock Solutions 2:1. Mix 2 parts of DI water with 1 part of Tertiary Stock Solution.

### **TERTIARY NUTRIENT STOCK SOLUTIONS**

The Tertiary Stock Solutions are prepared for seawater enrichment in 300 liter mass culture tanks only. The Nitrate/Phosphate and Silicate Tertiary Stock Solutions are prepared from reagent grade chemicals. The Vitamin and Trace Metal Tertiary Stock Solutions are prepared from the Primary Stock Solutions. For an F/2 strength enrichment, use 1 ml of each nutrient solution/3 liters of seawater. An F/4 strength enrichment (1 ml of each nutrient solution/6 liters of seawater) can be used after initial inoculation to supplement the culture.

#### Preparation of Tertiary Nitrate/Phosphate Stock Solution

1. Dissolve 900 g of sodium nitrate and 60 g of sodium phosphate in 4 liters of DI water. Refrigerate in a Nalgene container.

2. Date and label as: **“Tertiary nitrate/Phosphate Stock Solution for Mass Culture Tanks. Use 1 ml nutrient solution/3 liters of seawater for F/2 strength.”**

#### Preparation of Tertiary Silicate Stock Solution

1. Dissolve 360 g of sodium silicate in 4 liters of DI water. Refrigerate in a Nalgene container.
2. Date and label as: **“Tertiary Silicate Stock Solution for Mass Culture Tanks Use 1 ml nutrient solution/3 liters of seawater for F/2 strength.”**

#### Preparation of Tertiary Vitamin Stock Solution

1. Defrost one 60 ml portion of frozen Vitamin Primary Stock Solution. Mix with 3.940 liters DI water. Refrigerate in a Nalgene container.
2. Date and label as: **“Tertiary Vitamin Stock Solution for Mass Culture Tanks. Use 1 ml nutrient solution/3 liters of seawater for F/2 strength.”**

#### Preparation of Tertiary Trace Metal Stock Solution

1. Dissolve 37.8 g of ferric chloride, 52.2 g of EDTA and 12 ml of each of the five primary Trace Metal Solutions in 4 liters DI water. Refrigerate in a Nalgene container.
2. Date and label as: **“Tertiary Trace Metal Stock Solution for Mass Culture Tanks. Use 1 ml nutrient solution/3 liters of seawater for F/2 strength.”**

### **TUBE/FLASK ENRICHED SEAWATER MEDIA**

The Secondary and Tertiary Stock Nutrient Solutions are prepared with DI water and are used to enrich seawater directly in the carboys and mass culture tanks, respectively. The volume of each nutrient solution needed to enrich the seawater in a flask or tube culture, however, is so small (0.05 ml of nutrient solution per 50 ml flask) that it is inefficient to add nutrients to seawater in these vessels. Therefore, large quantities of enriched seawater media are prepared (one with silicates and one without silicates) with which the flasks and tubes are filled directly.

#### Preparation

1. Add 4 ml of each of the Secondary Nitrate/Phosphate and Trace Metal Stock Solutions to 2 liters salt water. Add silicates to one jar only. Mix well. Adjust volume to 4 liters. **DO NOT ADD VITAMINS (SEE NOTE BELOW)**. Cap with foil; leave stir bar in each jar.
2. Date and label each jar as: **“Enriched Seawater Media, with Silicates, for Tubes & Flasks”** or **“Enriched Seawater Media, without Silicates, for Tubes and Flasks.”**
3. Refrigerate. Mix well before use.

NOTE that Vitamins are not added to the Enriched Seawater Media. Vitamins must be autoclaved separately at a pH of 4.0-5.0. Prepare vitamins for autoclaving by adding 50-100 ml of Secondary Vitamin Stock Solution to a clean 125 ml flask. Normally the residual acid remaining on the inner glass surface after 3 rinses with DI water is sufficient to lower the pH of the Vitamin solution in the flask. If not, add a drop of nitric acid to the flask. Always check the pH before autoclaving. The flask, capped with foil, need only be autoclaved one time, and should be stored refrigerated.

#### LITERATURE CITED

Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates, pp. 29-60, IN: *Culture of Marine Invertebrates*, W.L. Smith and M.H. Chanley, eds., Plenum Publishing Corp., New York, New York.

McLachlan, J. 1973. Growth media - marine, pp. 25-51, IN: *Handbook of Physiological Methods*, J.R. Stein, ed., Cambridge University Press, London.

Stein, J.R. 1973. *Handbook of Physiological Methods*. Cambridge University Press, London.

#### FURTHER READING

Fox, J.M. 1983. Intensive algal culture techniques. IN: *CRC Handbook of Mariculture*, Volume 2: Crustacean Aquaculture, J.R. McVey, Ed.

Table 1: Six taxonomic classes of algae listing species used in mariculture.  
Starred species are those maintained in tube culture at Texas A&M.

---

Class Bacillariophyceae (diatoms):

- Chaetoceros calcitrans
- \* Chaetoceros gracilis
- Chaetoceros simplex
- Ditylum brightwellii
- \* Phaeodactylum tricornutum
- \* Skeletonema costatum
- \* Thalassiosira fluviatilis

Class Haptophyceae (golden brown flagellates):

- Coccolothus huxleyi
- \* Isochrysis galbana
- \* Isochrysis sp. (Tahitian strain)

Class Prasinophyceae (greenish-colored algae not belonging to the true green algae):

- Micromonas pusilla
- \* Tetraselmis chuii
- \* Tetraselmis suecica

Class Chlorophyceae (green algae):

- Chlorella autotrophica
- Chlamydomonas coccoides
- Dunaliella tertiolecta

Class Chrysophyceae (golden brown flagellates):

- Monochrysis sp.

Class Cryptophyceae (naked flagellates):

- Cryptomonas sp.
-

Table 2: Sources for obtaining algae stock cultures in the United States.

---

1. Indiana university Culture Collection (IUCC)  
Department of Botany  
Indiana University  
Bloomington, IN 47401
  2. American Type Culture Collection (ATCC)  
12301 Parklawn Drive  
Rockville, MD 20852
  3. Culture Collection of Algae (WHOI)  
Woods Hole Oceanographic Institution  
Woods Hole, MA 02543
  4. Culture Collection of Thermophilic Bluegreen Algae  
c/o Dr. R.W. Castenholz  
Department of Biology  
University of Oregon  
Eugene, OR 97403
  5. Carolina Biological Supply Company  
Burlington, NC 27215
  6. Wards Natural Science Establishment, Inc.  
P.O. Box 1712  
Rochester, NY 14603
  7. Provasoli-Guillard Center for Culture of Marine Phytoplankton  
Bigelow Laboratory for Ocean Sciences, McKnown Point  
West Boothbay Harbor, ME 04575  
(207) 633-2173
-

Table 4: Summary of ingredients used to prepare the six Primary, the four Secondary, and the four Tertiary Nutrient Stock Solutions.

Nutrient	Primary Stock Solution <sup>a</sup>	Secondary Stock Solutions <sup>b</sup>	Tertiary Stock Solutions <sup>c</sup>	
Nitrate	---	300g	900g	A Solution
Phosphate	---	20g	60g	
Silicate	---	120g	360g	
B-1	20g <sup>d</sup>	20ml	60ml	B Solution
Biotin	0.1g	(frozen	(frozen	
B-12	0.1g	portion)	portion)	
Iron	---	12.6g <sup>f</sup>	37.8 <sup>g</sup>	C Solution
EDTA	---	17.4g	52.2g	
Trace Metals:				
Copper	9.8g <sup>e</sup>	4ml	12ml	
Zinc	22.0g	4ml	12ml	
Cobalt	10.0g	4ml	12ml	
Manganese	180.0g	4ml	12ml	
Molybdate	6.3g	4ml	12ml	

<sup>a</sup>Used in the preparation of Secondary and Tertiary Stock Solutions.

<sup>b</sup>Used to enrich Tubes, Flasks and Carboys at F/2 strength (use 1ml nutrient/liter of seawater). Prepare each in a 4 liter volume.

<sup>c</sup>Used to enrich Mass Culture Tanks at F/4 strength (use 1ml nutrient/6 liter of seawater). Prepare each in a 4 liter volume.

<sup>d</sup>Mix all three vitamins in 1 liter DI. Freeze in 60ml and 20ml portions.

<sup>e</sup>Prepare each trace metal in a separate 1 liter volume of DI.

<sup>f</sup>Prepare a 4 liter volume with iron, EDTA and 4ml of each of the five Trace Metal Primary Stock Solutions.

A, B, and C are made and kept separately to ensure stable conditions while in storage

Table 5: Chemical formulas of compounds used to prepare nutrient stock solutions and purchasing information.

Compound	Chemical Formula	Catalogue No. (source)
Sodium Nitrate (granular)	$\text{NaNO}_3$	S-342 (a)
Sodium Phosphate (granular, monobasic)	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	S-468 (a)
Sodium Silicate (metasoluble)	$\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$	S-407 (a)
Thiamine Hydrochloride (B-1)	---	4700-100 (a) EK-05180-5 (b) 103028 (d)
Biotin	---	1368468 (a) EK-14838-7 (b) 101023 (d)
Cyanocobalamin (B-12)	---	1368463 (a) EK-14636-7 (b) 103271 (d)
Ferric Chloride	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	I-88 (a)
EDTA (Disodium Ethylene-diamine Tetraacetate)	$\text{Na}_2\text{C}_{10}\text{H}_{14}\text{O}_8\text{N}_2 \cdot 2\text{H}_2\text{O}$	S-311 (a)
Cupric Sulfate (fine crystal)	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	C-493 (a) JT1843 (b)
Zinc Sulfate (crystal)	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	Z-68 (a) 8880-1 (c)
Cobalt Chloride (crystal)	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	C-371 (a) JT1670-4 (b)
Manganese (ous) Chloride (crystal)	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	M-87 (a) JT2540-4 (b)
Sodium Molybdate (powder)	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	S-336 (a) JT3764-1 (b)
Chlorox (5.25% Sodium Hypochlorite)	---	Kroger Brand
Sodium Thiosulfate (anhydrous)	$\text{Na}_2\text{S}_2\text{O}_3$	S-446 (a)

<sup>a</sup>Fisher Scientific, 10700 Rockley Rd., P.O. Box 1307, Houston, TX 77251, (800)392-3307.

<sup>b</sup>VWR Scientific, P.O. Box 33348, Houston, TX 77033, (800)392-3338.

<sup>c</sup>American Scientific, 4660 Pine Timbers, Suite 100, Houston, TX 77041, (800)392-3338.

<sup>d</sup>ICN Nutritional Biochemicals, 26201 Miles Rd., Cleveland, OH 44128, (216)831-3000.

## ALGAE APPENDIX

### Disinfection

Clorox (5.25% hypochlorite solution) 0.08 ml/L culture water

Thiosulfate STOCK 0.22 ml/L culture water

(Prepare sodium thiosulfate stock solution by dissolving 2 g of sodium thiosulfate in 200 ml of DI water.)

or

0.0275g thiosulfate/ml clorox (0.04 estimate in the lab per ml clorox)

NOTE: Chlorine level must be checked this is a conservative number.

### Carboy (18 L)

Clorox 1.4 ml

Thiosulfate STOCK 4.0 ml

### Mass culture (40L)

Clorox 3.2 ml

Thiosulfate STOCK 8.8 ml

### Reservoir (1,600 L)

Clorox 64 ml

Thiosulfate 22 g

Note: if ciliate problem increase Clorox by 2X