

IRON REDUCTION AND MICRONUTRIENT NUTRITION  
OF JUVENILE MACROCYSTIS PYRIFERA (L.) C. A. AGARDH (GIANT KELP)  
DETERMINED BY A CHEMICALLY DEFINED MEDIUM, AQUIL

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## ABSTRACT

Iron reduction and micronutrient nutrition of juvenile Macrocystis pyrifera (L.) C. A. Agardh (giant kelp) were investigated using a chemically defined artificial seawater medium, Aquil. Juvenile sporophytes were grown and Aquil formulations were prepared in a Class 1000 cleanroom. Weights of the juveniles at the beginning and end of experiments determined the success of the various Aquil formulations.

Micronutrient experiments showed that kelp store surprisingly large quantities of Fe, Mn, Cu, Zn, Mo, Co and I, enough for 20- to 30-fold increases in weight. A maximum growth rate of 0.38 per day was measured for juvenile Macrocystis grown in Aquil. X-ray fluorescence analysis of dried, pressed blades determined kelp tissue element concentrations.

Kelp grew fastest with 250 nM Fe, 5 nM Mn, 100 nM Zn, 1000 nM Mo and 300 nM Co. Concentrations of iodine up to 500 nM  $I^-$  and of copper up to 500 nM produced neither stimulating nor inhibitory responses. Theoretical chemical species concentrations in Aquil formulations were calculated using the thermodynamic equilibrium computer program REDEQL2.

Kelp reduced Fe(III) to Fe(II) supporting the hypothesis that prior to uptake, iron is reduced in and then dissociates from the chelate complex, Fe-EDTA. This mediation was enhanced by the presence of light. The effects of photoreduction alone on Fe(III) reduction were studied, establishing its minor contribution as a source of Fe(II).

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## NOTATIONS

EDTA	Ethylenediamine tetraacetate.
BPDS	4, 7-di (4-phenylsulfonate)-1, 10- phenanthroline, (bathophenanthroline disulfonic acid).
FEP	Fluoroethylene polymer.
TFE	Tetrafluoroethylene.
TCE	Trichloroethylene.
lpe	Linear polyethylene.
L	Liter.
mL	Milliliter.
M	Moles per liter.
uM	Micromoles per liter.
nM	Nanomoles per liter.
umoles	Micromoles.
nm	Nanometer.
g	Mass unit, grams.
sec	Time unit, seconds.
<u>ug-at g</u> dry wt.	Microgram - atoms per gram dry weight.
$A_n$	Absorbance of media for condition n over wavelengths 534 to 536 nm, absorbance units.
R	Specific growth rate of juveniles, per day.
R'	Specific growth rate of juveniles, at 10°C, per day.
B	Dimensionless temperature factor to convert R to R'.



## NOTATIONS (Continued)

$W_i$	Initial wet weight of juveniles on Day 1 of experiment, grams.
$W_f$	Final wet weight of juveniles on final day of experiment, grams.
$t$	Time unit, days.
Q-water	Ultrapure water designed to be free of biological, ionic, particulate and organic impurities.
Aquil SB	Artificial seawater, Aquil, containing the standard background concentrations of micronutrients.
XRF	X-ray fluorescence.
AAS	Atomic Absorption Spectrophotometry.
LPI	Low Pressure Impactor.
PIXE	Particle Induced X-ray Emission.
$\Delta$	Change in total number of moles of a metal in one flask at the end of an experiment to account for XRF data, moles.

## CHAPTER 1

## INTRODUCTION

1.1 Project Selection

This thesis research was part of a larger project investigating biomass production in oceanic environments as a possible alternative energy source. A variety of physical, chemical and biological phenomena were under investigation. Success of the oceanic energy farm concept ultimately depends on economic feasibility. Economics, in turn, is very sensitive to productivity and yield by the crop of plants (biomass). Productivity is intimately related to proper nutrition of the crop, which is the topic of this thesis.

Giant kelp, Macrocystis pyrifera, was chosen as the experimental species to represent the crop. Macrocystis growth in large aquaria ranged from satisfactory to excellent in a medium of deep ocean water (North, 1978a). Variation in trace metals may have caused some of the differences in growth. A complete understanding of nutritional requirements was needed to optimize growth rates. In particular, micronutrients iron, manganese, copper, zinc, molybdenum, iodine and cobalt were presumably critical for kelp nutrition (Kuwabara, 1980). Special emphasis was placed on iron reduction by Macrocystis.

## 1.2 Previous Research

### 1.2.1 Iron

Iron occurs in the ocean as a trace metal and is ubiquitous in the air and soil as well as water. It is important in metabolic processes of terrestrial and marine organisms. Iron is required by virtually all organisms. It functions as an electron carrier in oxidation-reduction reactions in cytochromes and ferridoxin (Lehninger, 1975). Iron exists primarily in the ferric ( $\text{Fe}^{3+}$ ) state in aerobic marine environments and forms highly insoluble hydroxides and phosphates. Iron exists at low concentrations in the ocean. Total iron was reported to be 89 nM, and was formerly thought to exist largely as colloidal ferric hydroxide,  $\text{Fe}(\text{OH})_3$  (Atkinson and Stefansson, 1969). It now appears that half of the total dissolved iron is bound by organic matter (Sugimura *et al.*, 1978).

North (1980) found that iron in nearshore surface ocean water off California was in a lower range from 10 to 30 nM. Concentrations of iron in algal culturing media have ranged from 200 nM (Müller, 1962) to  $1.8 \times 10^5$  nM (Manahan and Smith, 1973). EDTA and other chelates were routinely used to keep iron dissolved. Previous research concerning iron reduction is discussed in Chapter 3.

### 1.2.2 Manganese

Manganese is essential to algae, participating in the  $O_2$ -evolving system of photosynthesis as an activator of many enzymes acting on phosphate-containing compounds. Manganese also has additional functions in enzyme catalysis (Chapman, 1979; Brock, 1974). Arginase and phototransferease contain manganese as  $Mn^{2+}$  (Lehninger, 1975). Manganese occurs in several pools in algal cells, two-thirds of which is weakly membrane-bound in chloroplasts, with the remaining one-third tightly bound (Sauer, 1980).

Manganese exists in seawater both as manganous ( $Mn^{2+}$ ) complexes with chlorides, and as higher oxidation states, principally  $Mn^{4+}$ , forming insoluble  $MnO_2$ . Bender et al (1977) determined that manganese exists primarily as a dissolved species at 18.2 nM, while Murray and Brewer (1977) report 3.6 nM. North (1980) found a range of 5 to 40 nM Mn for nearshore surface ocean water. Manganese values for seawater ranged from 5 to 22 nM when ultraclean techniques were used by Knauer (1978), who also found phytoplankton photosynthesis was inhibited at 20 nM.

North (1978c) found that seasonal fluctuations in manganese concentration of water from a depth of 300 m correlated with fluctuations in growth rates of large juvenile Macrocystis. Additions of manganese and sometimes iron to water from 300 m deep sometimes increased Macrocystis growth (North, 1978b). North hypothesized that manganese carried by terrestrial runoff was communicated rapidly to deep

water. Sedimentation eventually reduced the manganese in deep water to undetectable levels. Calculations by Bender et al. (1977) implied that rate of buildup by manganese in sediments is too high to be accounted for solely by  $MnO_2$  sedimentation from biological and atmospheric inputs to the ocean and must also include input from terrigenous particles.

### 1.2.3 Copper

Algal chloroplasts contain a blue copper-protein, plastocyanin (Lehninger, 1975). Copper plays a role in certain oxidation-reduction enzymes such as tyrosinase and cytochrome oxidase. Copper is thus a required metal and probably performs essential roles in photosystem I activity in all algae (Chapman, 1979).

Literature reports on copper toxicity in algae usually are unaware of possibilities of high copper levels due to contamination. Distilled water at one laboratory contained about 4700 nM Cu so that media and tracer solutions prepared with this water might easily misjudge toxicity (Steeman Neilsen and Kamp-Nielsen, 1970). Steeman Neilsen and Wium-Andersen (1972) further found that a very low copper concentration of 90 nM inhibited photosynthesis in Nizschia palea. Strömngren (1979) found that 12,600 nM copper was lethal to the brown alga Ascophyllum nodosum. North (1978a) reported that 100 nM copper inhibited growth of large Macrocystis juveniles in enriched seawater.

Hutchinson (1973) found that the growth of the green microalga Haematococcus capensis was initially stimulated by additions of up to 1600 nM Cu. Apparently free cupric ion is the actively toxic form (Sunda and Guillard, 1976). Anderson and Morel (1978) calculated that  $\text{Cu}^{2+}$  inhibited motility of the dinoflagellate Gonyaulax tamarensis by 50% at 0.04 nM.

Martin (1967) suggested that algae excrete complexing ligands to regulate concentrations of free copper. Van den Berg et al. (1979) found that complexing capabilities of various algal exudates when added to a culture of Chlorella vulgaris ameliorated copper toxicity at concentrations as high as 3148 nM. Swallow et al. (1978) found that only one out of eight phytoplankton species excreted copper complexing materials.

According to Murray and Brewer (1977) copper exists in the ocean at 8 nM primarily as  $\text{CuOH}^+$ . North (1980) found nearshore values in southern California of 3 to 10 nM Cu while Boyle et al. (1977) reported 1.5 nM Cu in the California current near Monterey. Windom and Wallace (1980) found copper varied seasonally from 0.078 to 1.6 nM for nearshore Florida.

#### 1.2.4 Zinc

Zinc plays a structural role in certain enzymes by holding protein subunits in proper configurations for enzyme activity (Brock, 1974). These enzymes include alcohol dehydrogenase, carbonic anhydrase and carboxypeptidase (Lehninger, 1975). Zinc is required by all algae and plays a part in RNA formation (Chapman, 1979).

Zinc is ubiquitous so that reliable data require strict precautions against contamination. Bruland, Knauer and Martin (1978) used ultraclean techniques and found zinc occurred at very low levels of 0.15 to 9 nM in the open ocean. Coastal waters appear to have a much higher level, about 10 to 50 nM (North, 1980).

The brown alga Laminaria digitata showed toxic effects on growth from 1530 nM zinc (Bryan, 1969). Eide and Myklestad (1980) suggested that uptake and release of zinc required an input of metabolic energy. Alginic acid, the most important polysaccharide in brown algae, has a low affinity for zinc yet tissues concentrate the metal highly from seawater (Bryan, 1969; Myklestad et al., 1978). Toxicity from 30,595 nM zinc towards diatoms Phaeodactylum tricorutum was relieved by addition of polyphenols from the brown alga Ascophyllum nodosum (L.) Le Jolis (Ragan et al., 1980). Strömberg (1979) found that 21,416 nM zinc in seawater reduced Ascophyllum growth 25% while 214,165 nM reduced growth 60 to 80%. North (unpublished) found that 1000 nM zinc added to either ocean water from 300 m depth or from the surface (zinc background of 100 nM) reduced Macrocystis growth rates.

### 1.2.5 Cobalt

The trace element cobalt is a constituent of vitamin B<sub>12</sub> and is required by some seaweeds (Brock, 1974; Chapman, 1979). The metabolism of cobalt is unknown.

Yamamoto (1965) demonstrated that 17 species of brown seaweeds concentrated cobalt, and North (1977) showed that was also true for large Macrocystis juveniles. The range of concentrations of cobalt used for enriching media has been very large. Toerin et al. (1971) used only 20 nM, while Chu (1942) added 4200 nM. In 1968, Provasoli recommended the addition of cobalt as 620 nM.

Cobalt exists in seawater at 0.8 nM (Murray and Brewer, 1977). Stumm and Brauner (1975) have calculated that free cobalt (Co<sup>2+</sup>) is the major species. Work by Robertson (1970) showed a surface seawater cobalt concentration of 0.13 nM for northcentral Pacific Ocean and 1.1 nM for northeastern Pacific Ocean off the coast of Oregon. A concentration of 5 nM measured at Newport, Oregon, represented high cobalt contribution from terrestrial drainage.

Norris and Kelly (1976) found that the yeast Saccharomyces cerevisiae accumulated cobalt in a two-stage process. First the cation was bound to the cell surface by an energy independent pathway followed by a metabolism-dependent progressive uptake of large amounts of the cation. Severest inhibition of cobalt uptake involved cations of similar size, such as Zn<sup>2+</sup>, Ni<sup>2+</sup>, Mg<sup>2+</sup> and successively less by Mn<sup>2+</sup>.



### 1.2.6 Molybdenum

Certain enzymes, molybdoflavoproteins, contain both molybdenum and cytochrome (Brock, 1974). The enzyme nitrate reductase which catalyzes the first step in nitrate utilization, is widely distributed in plants and fungi (Lehninger, 1975). Murray and Brewer (1977) reported that molybdenum exists in seawater at 100 nM in the ionic form  $\text{MoO}_4^{2-}$ .

### 1.2.7 Iodine

The metabolic role of iodine in seaweeds is unknown. Iodine is required by several red and brown algae, and up to 1% of the dry weight of kelps may be iodine (Chapman, 1979).

Jacques and Osterhout (1938) found that marine algae concentrated iodine highly, and suggested that iodine was actively accumulated from the sea. Roche and Yagi (1952) showed by tracer studies that both organic and inorganic forms of iodine exist in bull kelp, Nereocystis luetkeana. A concentration of 400 nM KI stimulated the brown alga Laminaria hyperborea, while  $4 \times 10^5$  nM was inhibitory (Harries, 1932). Hsiao (1969) found that iodine was an essential element for the growth, morphogenesis and reproduction of the marine brown alga Petalonia fascia cultured in synthetic seawater. Zoospores exhibited different morphologies prior to blade development, depending on iodine concentrations ranging from 4000 nM to  $4 \times 10^6$  nM KI. Slight inhibition occurred at  $20 \times 10^6$  nM, a concentration 50,000 times

greater than found in seawater. Codomier et al. (1979) obtained maximal growth of the red alga Asparagopsis armata at 5000 nM of either iodide or iodate in natural seawater and inhibition at 15,000 nM iodide or iodate.

According to Murray and Brewer (1977) iodine was 500 nM in the ocean. Iodine exists in seawater as two major forms, iodide ( $I^-$ ) and iodate ( $IO_3^-$ ). Wong and Brewer (1976), using neutron activation analysis, found 75 nM  $IO_3^-$  and 260 nM  $I^-$  in surface seawater for a combined total of 335 nM I.

#### 1.2.8 Biologically Active Forms of Micronutrients

Barber and Ryther (1969) examined the chelating capacity of newly upwelled ocean water. They found that addition of EDTA or a "zooplankton extract" enhanced phytoplankton growth, while addition of trace metals significantly decreased growth. They suggested that upwelled water ages under the influence of organic chelators released by surface organisms. Steemann Neilsen and Wium-Andersen (1970) proposed that organic conditioning reduces copper toxicity. Whether the explanation of plant nutrition due to metal chelation involves either increased availability or a reduction of toxicity, the metal's chemical form and not its total amount controls biological activities.

Interactions involving micronutrients in natural seawater are so complex that it is not possible to define their chemical states. Total amounts of the components in synthetic seawater media can be defined and

controlled, enhancing reproducibility of results from laboratory experiments. Ligands such as EDTA can be added to artificial seawater forming highly stable, soluble chelate complexes and keeping highly insoluble metals such as iron in solution. This technique is used extensively in nutrient solutions for terrestrial and aquatic plants.

### 1.3 Rationale for this Study

The ranges of concentrations of micronutrients used as enrichments and reported in previous research as causing algal toxicities in synthetic seawater or natural seawater are very large. Contamination from chemical reagents used to prepare media and from particles in the atmosphere have almost certainly complicated many experiments and probably explain variations seen in the literature. The unknown interactions between micronutrients and organics in natural seawater prevent predictions of speciation and possible metal precipitation in the media. It has been hypothesized that iron is reduced and dissociates prior to uptake, but this has not been adequately proven for Macrocystis.

These difficulties complicated almost all literature comparison and interpretation. Only a few microalga have been examined by studies utilizing rigorous chemically clean conditions. Research on a macroalga was needed involving experiments conducted with strict precautions as to macro- and micronutrient contamination of media and the test organism. Such an investigation would provide a more accurate understanding of minimal amounts of micronutrients required, working at the low end of

the range of concentrations used. At the high end of the concentration scale, the investigations might yield precise definition of toxic thresholds for those micronutrients capable of causing inhibitory effects. The need for establishing nutritional requirements of the Macrocystis sporophyte provided the opportunity for examining effects from exposures to wide ranges of concentrations of the critical micronutrients, using rigorous cleanroom techniques.

## Chapter 2

## EXPERIMENTAL CONDITIONS

2.1 Laboratory Conditions

Experiments were conducted at the W. M. Keck Engineering Laboratory at the California Institute of Technology in Pasadena, California, beginning in 1976.

2.1.1 Cleanroom System

Rigorous precautions were taken with the physical arrangement of the cleanroom, use of pure reagents, and cleaning and storage of apparatus to guard against possible metal contamination.

Nomad vinyl fiber mats (3M Co.) were placed outside the laboratory door to remove and trap dust within the several layers of material. Shoes were removed before entering. The first of two sets of Tacky-Mats (Tackmer Corp.) followed, to strip particles from soles of the feet. These mats continued under the laboratory door into the cleanroom antechamber. Rubber stripping on the door bottom prevented air intrusion into the laboratory. After closing the door, one proceeded through a set of polyethylene flaps into a second antechamber where lab coat and shoes were stored. A second set of polyethylene flaps led to the cleanroom area.

Wood structures covered by polyethylene sheeting formed the dropped ceiling, antechamber walls and flaps, as well as flaps at the cleanroom's far end leading to a fume hood section. Metal shelves and fixtures were removed and all exposed metals, such as nail heads, were sealed with GE RTV sealant. Electrical outlets were sealed with plastic plugs to prevent air intrusion from wall spaces. Floors and walls were painted with two coats of metal-free epoxy Rust-Mate paint (Zynolyte Products Corp.).

An eight-inch diameter feather fan provided positive pressure in the laboratory to prevent outside air intrusion each time the door was opened. Incoming air was originally filtered through two layers of polyester batting (Fram filter). A finer filter system (high efficiency particle air (HEPA) filter, from Laminaire Corp., with a three-layer Fram prefilter) was adopted after severe cleanroom contamination occurred (an overnight deposition of 1/4" fine black particles.)

A cleanhood providing low-particle count laminar air flow was upgraded to prevent resuspension by dust particles from work areas into the air stream, thence possibly contaminating open liquids or kelp. This hood was permanently lined with FEP overlay (Cole-Parmer Instrument Co.) which in turn was covered with disposable sheets of Handiwrap (Dow Chemical Co.) plastic film.

Particle counts were recorded for incoming air, laboratory benches, laminar flow hood, fume hood and floor with a RoyCo optical particle

counter (Model #245). Particle levels calculated prior to the HEPA installation fell by 93% compared with values afterwards. The laminar flow hood yielded 746 particles per cubic foot of air ranging from 0.56 to 8 nm diameter, which corresponded to Class 1000 conditions.

Acids were stored and diluted in the fume hood, which was protected by epoxy paint, then lined with teflon overlay. Handles and spigots on all gas and water fixtures in the fumehood and laboratory were stripped of old paint with boiling nitric acid, revealing the original bakelite finish. The telephone was stored in a plastic box to prevent possible released copper contamination. Magnetic stirrers and other necessary metal equipment were painted with epoxy or covered with teflon overlay, and sealed in plastic bags. Fram filters throughout the laboratory were replaced when discolored. Floors were washed with Q-water and Scott Utility Wipes.

#### 2.1.2 Q-Water

All cleaning and aqueous solutions employed the ultrapure water from the Milli-Q Water Purification System (Millipore Corporation), called "Q-water". Distilled water was passed through series of columns to remove biological, particulate, organic and ionic (i.e. metal) contaminants. Electrical resistivity of all Q-water used was 18 megohms/cm-sec. Q-water was collected at the Kerckhoff Marine Laboratory, Corona del Mar, California, in twenty-liter acid-cleaned linear polyethylene carboys, and transported to Pasadena.

## 2.2 Cleaning Procedures

Besides atmospheric deposition, attention was paid to several other possible sources of contamination. Untalced Vanlab Poly gloves (VWR Scientific, Inc.) were worn at all times to prevent transferring oils and dust to apparatus. All glassware (pyrex and quartz), teflon (FEP and TFE) and linear polyethylene (lpe) were initially cleaned for three weeks to remove contaminants and leach impurities from the materials. Following each use, a four-day cleaning procedure was employed (Moody and Lindstrom, 1977).

### 2.2.1 Initial Three-Week Cleaning Procedure

All acids used for cleaning were reagent grade, diluted with Q-water, and stored in acid-cleaned twenty-liter polyethylene carboys. Equipment fashioned in the shop was wiped with TCE and Scott Microwipes tissues to remove grease. Otherwise, apparatus was first rinsed and then soaked for one week in 50% grade HCl. It was again rinsed once with 50% HCl, twice with Q-water, once with 50% HNO<sub>3</sub>, and soaked for one week in 50% HNO<sub>3</sub>. This was followed by two rinses with Q-water, then soaked in Q-water for at least one week. Apparatus not immediately used was wrapped in plastic film and stored in cleanroom cupboards. Sheets of polyethylene film were taped along the tops of all storage areas to prevent dust intrusion when opened.

### 2.2.2 Four-Day Acid Cleaning Procedure

All labware received a minimum four day cleaning procedure following each use. This involved rinsing then soaking at least three



days in 4N HNO<sub>3</sub>, then rinsing twice with Q-water, soaking at least one day in Q-water, with a final rinse in Q-water and stored.

### 2.2.3 Tygon Tubing

Tygon tubing transported filtered air to and away from the flasks containing kelp, never contacting media. Tygon tubing was rinsed four times with Q-water prior to use because it decomposes in acids.

### 2.3 Aquil Production

All culturing utilized the artificial seawater Aquil to enhance experimental reproducibility and permit precise chemical definition. Aquil was developed by Morel *et al.* (1979) for biological studies using low levels of trace metals. Ligands such as EDTA are added to keep highly insoluble metals such as iron in solution as stable, dissolved chelate complexes. The technique has been used extensively in nutrient solutions for terrestrial and aquatic plants.

The Aquil schematic developed by Morel was further adapted (Figure 2.1). The pH of Aquil was directly measured and adjusted if necessary. Morel's scheme did not include pH verification. Atomic adsorption analysis performed by Caltech technician Sylvia Garcia showed that chelex columns of ion-exchange resin became measurably less efficient at removing metals from the background salts solution after 10 L were eluted. Chelex was rejuvenated for reuse after 5 L were eluted. Morel replaced his after 25 L of elution or when the column became discolored or clogged. Cobalt was used instead of vitamin B<sub>12</sub>. Silica and

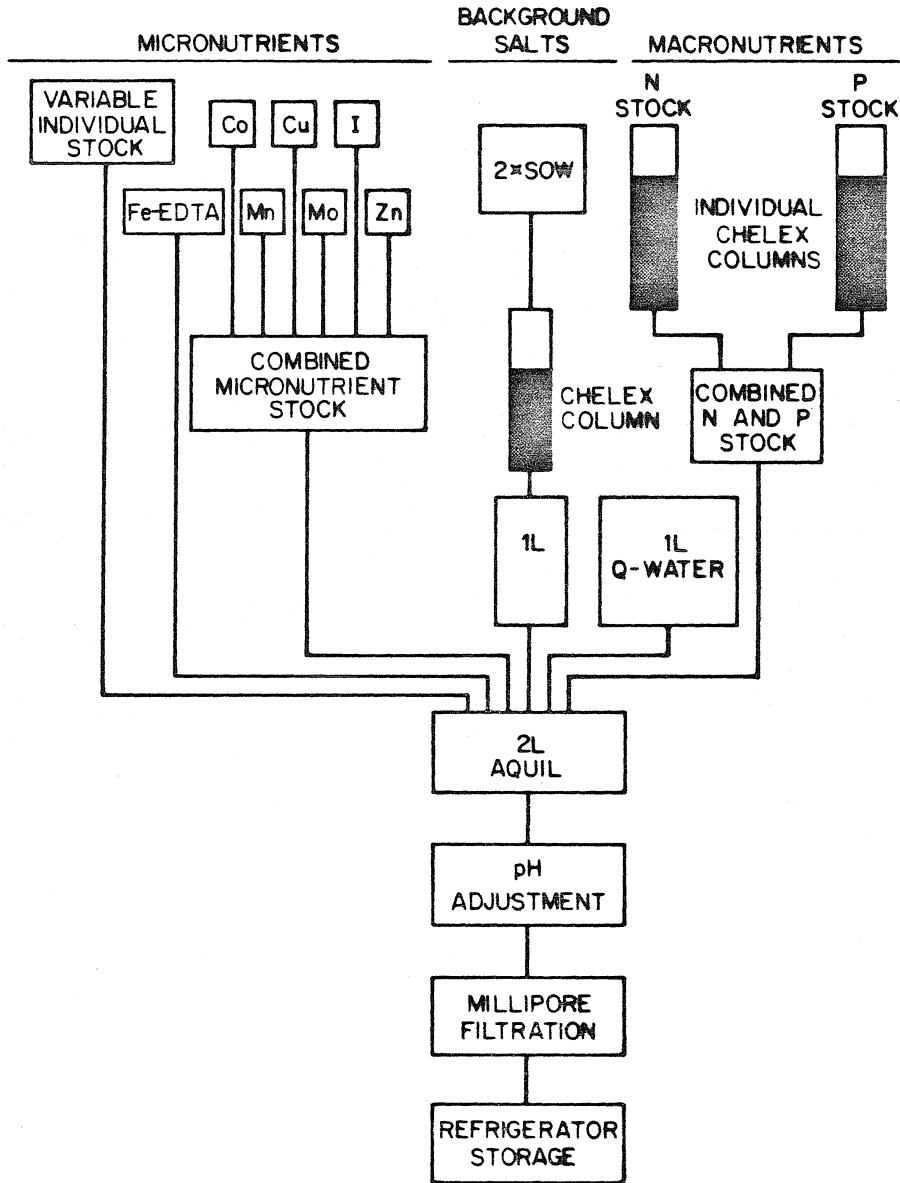


Figure 2.1 Schematic of the production of the synthetic seawater Aquil.

vitamins biotin and thiamine were not added. A background salt solution at twice the normal concentration was used because a larger supply could be prepared at one time, as very large quantities of Aquil were necessary for juvenile kelp culturing experiments. After elution, the salt solution was diluted to normal concentrations with Q-water.

### 2.3.1 Calculation of Aquil Speciation

When speciation of chelators and metals is known, micronutrient levels in Aquil can be effectively manipulated, based on thermodynamic considerations. The computer program REDEQL2 (Morel and Morgan, 1972; McDuff and Morel, 1973) was used to calculate theoretical concentrations of different chemical species in Aquil. In Aquil SB, for example, manganese exists partially as a free ion, while iron is almost totally bound to EDTA. Table 2.1 shows the composition and speciation of Aquil SB, which contains the standard background levels of metals and ligands. REDEQL2 computations indicated that an EDTA : Fe molar ratio of 20.5:1 would keep iron in a totally dissolved form and satisfy EDTA complexation with other metals. Speciation changed only slightly for other micronutrients when the manganese concentration was changed from 10 nM ( $10^{-8}$  M) in Aquil SB (Table 2.1) to 100 nM (Table 2.2) and to 0 nM (Table 2.3).

### 2.3.2 Background Salts

Background salts were first dissolved in 20 L of Q-water at a concentration twice that of standard ocean water (2 X SOW), (Table 2.4). One-liter aliquots of salt solution were eluted through columns of

Table 2.1 Composition and speciation of Aquil standard background (Aquil SB)

<u>Metal</u>	Analytical Concentration <u>-log [ Molar]</u>	Computed -log <u>[Molar Free Ion]</u>	Ligand of Computed Major Species or Free Metal Ion, Ligand (%), or Free Ion (%)
Calcium	1.98	2.11	Free ion (74)
Magnesium	1.26	1.33	Free ion (85), $SO_4^-$ (14)
Strontium	4.20	4.20	Free ion (100)
Potassium	1.99	2.01	Free ion (96.5)
Sodium	0.32	0.33	Free ion (98.6)
Iron	6.60	19.24	EDTA (100)
Manganese	8.00	8.91	EDTA (60), $Cl^-$ (26), Free ion (12.4)
Copper	8.30	13.69	EDTA (99.2)
Zinc	7.00	10.18	EDTA (99.5)
Cobalt	7.40	9.89	EDTA (99.2)
<u>Ligand</u>			
Carbonate	2.62	4.90	$Ca^{+2}$ (72)
Sulfate	1.54	1.89	Free ion (44), $Mg^+$ (27), $Na^+$ (24)
Chloride	0.25	0.25	Free ion (100)
Fluoride	4.15	4.38	Free ion (59), $Mg^+$ (40)
Bromide	3.08	3.08	Free ion (100)
Iodide	7.00	7.00	Free ion (100)
Phosphate	6.00	9.83	$H^+$ (50), $Mg^+$ (47)
EDTA	5.29	13.25	$Ca^+$ (91), $Fe^{+3}$ (5), $Zn^{+2}$ (2), $Mn^{+2}$ (0.1)
Borate	3.31	4.13	Free ion (15) $H^+$ (85)
Nitrogen	4.82	4.82	Free ion (100)
Molybdenum	7.00	7.00	Free ion (100)

Table 2.2 Composition and speciation of Aquil SB  
with 10x manganese concentration, 100 nM

	Analytical Concentration <u>-log [Molar]</u>	Computed -log <u>[Molar Free Ion]</u>	Ligand of Computed Major Species or Free Metal Ion, <u>Ligand(%), or Free Ion(%)</u>
<u>Metal</u>			
Manganese	7.0	7.90	EDTA(60), Cl <sup>-</sup> (26), Free ion (12), SO <sub>4</sub> <sup>-2</sup> (1.6)
Iron	6.6	19.23	EDTA (100)
Copper	8.3	13.68	EDTA (99.2)
Zinc	7.0	10.18	EDTA (99.5)
Cobalt	7.40	9.88	EDTA (99.2)
<u>Ligand</u>			
EDTA	5.3	13.25	Ca <sup>+2</sup> (90), Fe <sup>+3</sup> (5), Zn <sup>+2</sup> (2), Co <sup>+2</sup> (2), Mn <sup>+2</sup> (1.2)
Iodide	7.00	7.00	Free ion (100)
Molybdenum	7.00	7.00	Free ion (100)

Table 2.3 Composition and speciation of Aquil SB  
with 0 nM manganese concentration

	Analytical Concentration <u>-log [Molar]</u>	Computed -log <u>[Molar Free Ion]</u>	Ligand of Computed Major Species of Free Metal Ion, <u>Ligand (5), or Free Ion(%)</u>
<u>Metal</u>			
Manganese	-	-	-
Iron	6.6	19.24	EDTA (100)
Copper	8.3	13.69	EDTA (99.2)
Zinc	7.0	10.19	EDTA (99.5)
Cobalt	7.40	9.89	EDTA (99.2)
<u>Ligand</u>			
EDTA	5.3	13.25	Ca <sup>+2</sup> (91), Fe <sup>+3</sup> (5), Zn <sup>+2</sup> (2), Co (2)
Iodide	7.00	7.00	Free ion (100)
Molybdenum	7.00	7.00	Free ion (100)

Table 2.4 Composition of Aquil SB medium (with standard background micronutrient concentrations)

	Substance	<u>Initial Weight</u> g	<u>Initial Vol</u> ℓ	<u>Stock Conc</u> M	<u>Final Concentration</u> M
<u>Background Salts (2xSOW)</u>	NaCl	982.2	20	$8.39 \times 10^{-1}$	$4.20 \times 10^{-1}$
	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	444.0	20	$1.09 \times 10^{-1}$	$5.46 \times 10^{-2}$
	$\text{Na}_2\text{SO}_4$	163.6	20	$5.76 \times 10^{-2}$	$2.88 \times 10^{-2}$
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	61.6	20	$2.09 \times 10^{-1}$	$1.05 \times 10^{-2}$
	KCl	28.0	20	$1.88 \times 10^{-2}$	$9.39 \times 10^{-3}$
	$\text{NaHCO}_3$	8.0	20	$4.76 \times 10^{-3}$	$2.38 \times 10^{-3}$
	KBr	4.0	20	$8.32 \times 10^{-4}$	$4.16 \times 10^{-4}$
	$\text{H}_3\text{BO}_3$	1.2	20	$9.80 \times 10^{-4}$	$4.90 \times 10^{-4}$
	$\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$	0.68	20	$1.26 \times 10^{-4}$	$6.31 \times 10^{-5}$
	NaF	0.12	20	$1.41 \times 10^{-4}$	$7.14 \times 10^{-5}$
<u>Macronutrients</u>	$\text{KH}_2\text{PO}_4$	0.544	2	$2.0 \times 10^{-3}$	$1.0 \times 10^{-6}$
	$\text{NaNO}_3$	3.40	1	$4.0 \times 10^{-2}$	$2.0 \times 10^{-5}$
<u>Micronutrients</u>	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	1.705	1	$1.0 \times 10^{-2}$	$5.0 \times 10^{-9}$
	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.270	1	$1.0 \times 10^{-3}$	$2.5 \times 10^{-7}$
	$\text{Na}_2\text{EDTA}$	7.631	1	$2.0 \times 10^{-2}$	$5.1 \times 10^{-6}$
	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.396	2	$1.0 \times 10^{-3}$	$1.0 \times 10^{-8}$
	$\text{ZnCl}_2$	27.256	1	$2.0 \times 10^{-1}$	$1.0 \times 10^{-7}$
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	48.390	1	$2.0 \times 10^{-1}$	$1.0 \times 10^{-7}$
	KI	33.202	1	$2.0 \times 10^{-1}$	$1.0 \times 10^{-7}$
	$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	23.283	1	$8.0 \times 10^{-2}$	$4.0 \times 10^{-8}$

Biorad Laboratory Chelex 100 (an ion exchange resin to remove divalent cations), and diluted with Q-water in 2 L FEP teflon bottles, giving a final concentration of 1 X SOW. Acid cleaning of the 2 X SOW carboy was necessary in between batches to prevent precipitation by salts remaining from previous batches and adhering to carboy walls.

The chelex columns originally used were FEP teflon with TFE stopcock fittings. Problems such as short circuiting of flow by air bubbles, leaking of chelex from the column, and low flow rate because of small diameter, necessitated a change to glass chromatography tubes (Lab-Crest Scientific Glass Co.).

### 2.3.3 Addition of Micro- and Macronutrients

Micronutrients (trace metals and iodine) and macronutrients (nitrogen and phosphorus) were individually added to the freshly eluted and diluted SOW by micropipets with acid-cleaned plastic tips (Pipetman Models #P20D, P200D, P1000D). Compounds and concentrations of micro- and macronutrients are shown in Table 2.4. Stock solutions were stored in darkness at 5°C in 200 mL FEP teflon bottles. Small quantities of stock were transferred to 30 mL FEP teflon bottles before pipetting. Leftover stock was discarded to avoid contaminating the main stock bottle.

### 2.3.4 pH Adjustment

Aquil pH was adjusted to  $8.1 \pm 0.05$  with Baker ultrapure acid and base, Ultrex HCl and Ultrex  $\text{Na}_2\text{CO}_3$ . A Corning digital pH

meter (Model #130) with a glass microcombination electrode (Microelectrodes Inc., Model #MI-410) furnished direct pH readings of each 2 L batch of Aquil. The 1.5 mm diameter electrode tip just touched the liquid surface with an effective immersion depth of less than 1.2 mm in order to minimize possible Aquil contamination.

### 2.3.5 Filtering

Each 2 L batch of Aquil was filtered following pH adjustments through 0.45 um Millipore filters directly into two 1 L lpe bottles using a closed reaction vessel (Ace Glass, Inc.) and a hand-operated pump (VWR Scientific). Millipore filters were individually conditioned by filtering 300 mL of Q-water followed by 100 mL of the particular type of Aquil, which was then discarded. Apiezon stopcock grease was used to seal the reaction flask.

A MACE Corporation TFE teflon filter holder with a TFE 300 mL reservoir was initially used. Distortions in shape caused leaking around the filter, necessitating changes to the pyrex Millipore filter reservoir and holder (Model # XX10-147, Millipore Corp.), with ground glass filter support. After filtration, bottles containing Aquil were labelled, wrapped in plastic bags and sealed with tape, and stored in darkness at 5°C no longer than two weeks prior to use.

### 2.3.6 Combined Macronutrient Stock Solutions

Metal contaminants were removed from the two nitrate and phosphate stock solutions by elution through separate chelex columns



equilibrated to the solution's individual compound and ionic strength. The two solutions were then combined in an FEP bottle, and stored at 5°C in darkness.

### 2.3.7 Combined Iron and EDTA Stock Solution

Iron at  $10^{-3}$  M and 0.02 M EDTA were combined in one stock solution to reduce the time required for chemical equilibration and prevent possible iron hydroxide precipitation. Ultrex  $\text{Na}_2\text{CO}_3$  was added to  $\text{Na}_2\text{EDTA}$  in Q-water to produce a deprotonated EDTA. This form of EDTA readily complexed with Fe, added as  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . Precipitation problems at high iron levels were eliminated by the combined stock solution.

### 2.3.8 Individual Micronutrient Stock Solutions

Individual stock solutions were prepared for manganese, copper, zinc, cobalt, molybdenum and iodine. One mL of each individual stock was then added to a single liter of Q-water, yielding a combined metals stock solution. Micronutrient concentrations in the individual stocks were adjusted to produce the standard background concentrations after one mL of the combined solution was added to two liters of Aquil. Thus repetitive additions of individual micronutrients other than the individual element being investigated were eliminated. A different combined stock solution was prepared for each micronutrient tested, holding the other six micronutrients constant at their standard background levels.

### 2.3.9 Coulter Counter Tests

Aquil was tested for particles with Coulter Counter tests by Dr. James Hunt. New Aquil was extremely clean, while Aquil that had been used to grow kelp showed a significant but low level of particles. These centered in the 0.7 to 3.0 um diameter range. The 3.0 to 8.0 um range was much less predominant. Particles with diameters less than 1 um are too small to be algae, and were probably bacteria or organic exudates from the kelp that coagulated from bubbling action in the growth flask.

## 2.4 Experimental Culturing Equipment and Operations

Two factors governed design of the culturing equipment:

- A. controlling contamination from materials and air; and
- B. facilitating frequent visual inspections of the experiments as they progressed.

### 2.4.1 Growth Apparatus (Figure 2.2)

The growth apparatus was matching pyrex and quartz one liter flasks and bubblers fitted together with TFE teflon bushings. The gas dispersion tube was angled to one side to enhance circulation of the medium.

### 2.4.2 Growth Chamber (Figure 2.3)

The growth chamber was a converted Kelvinator freezer located in the cleanroom. Metal fixtures were removed and shelves were coated with rubber. Flasks were continuously illuminated from below with four

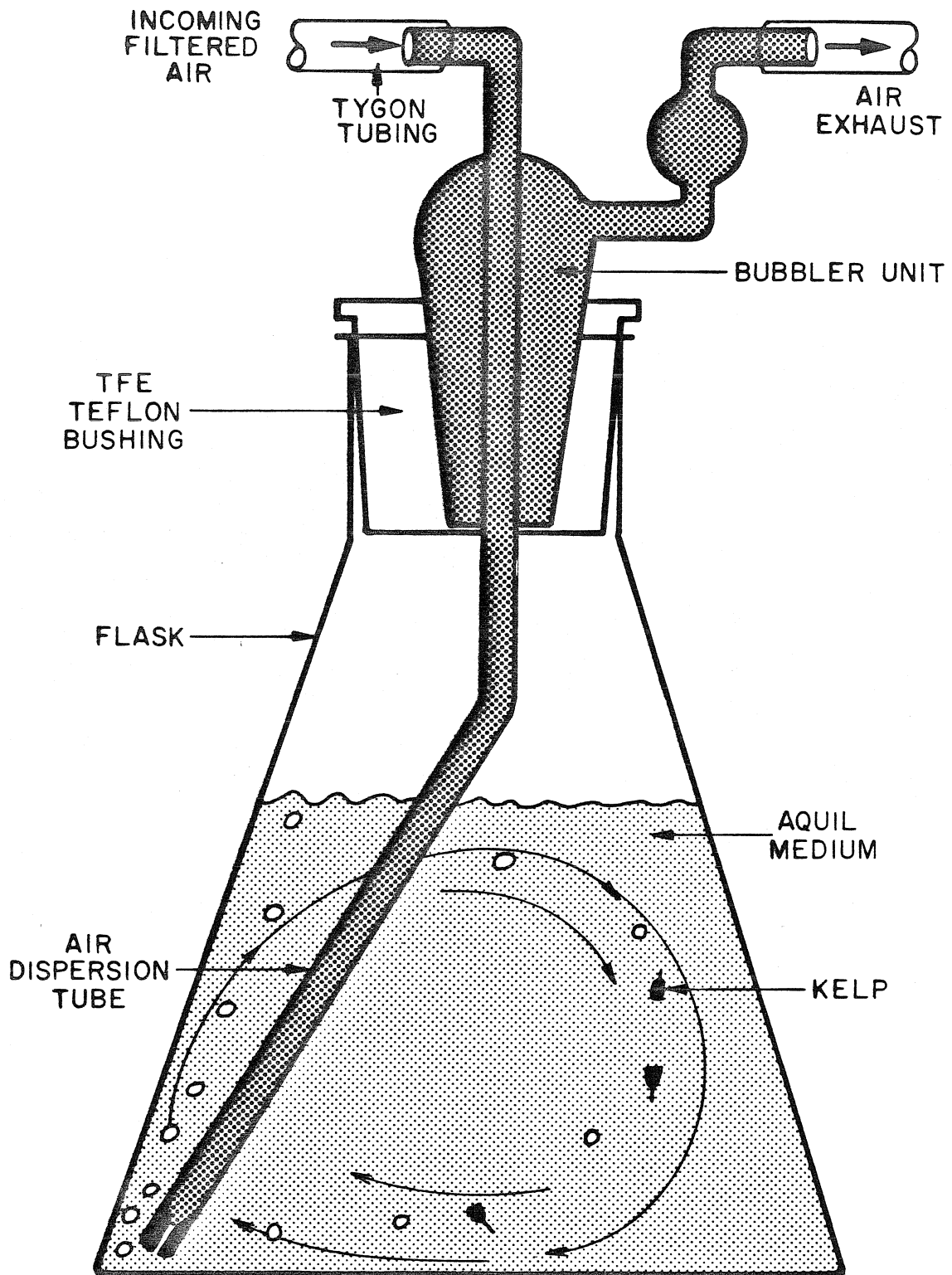


Figure 2.2 Growth apparatus used to test the effects of various Aquil formulations and iron reduction by juvenile Macrocyctis.

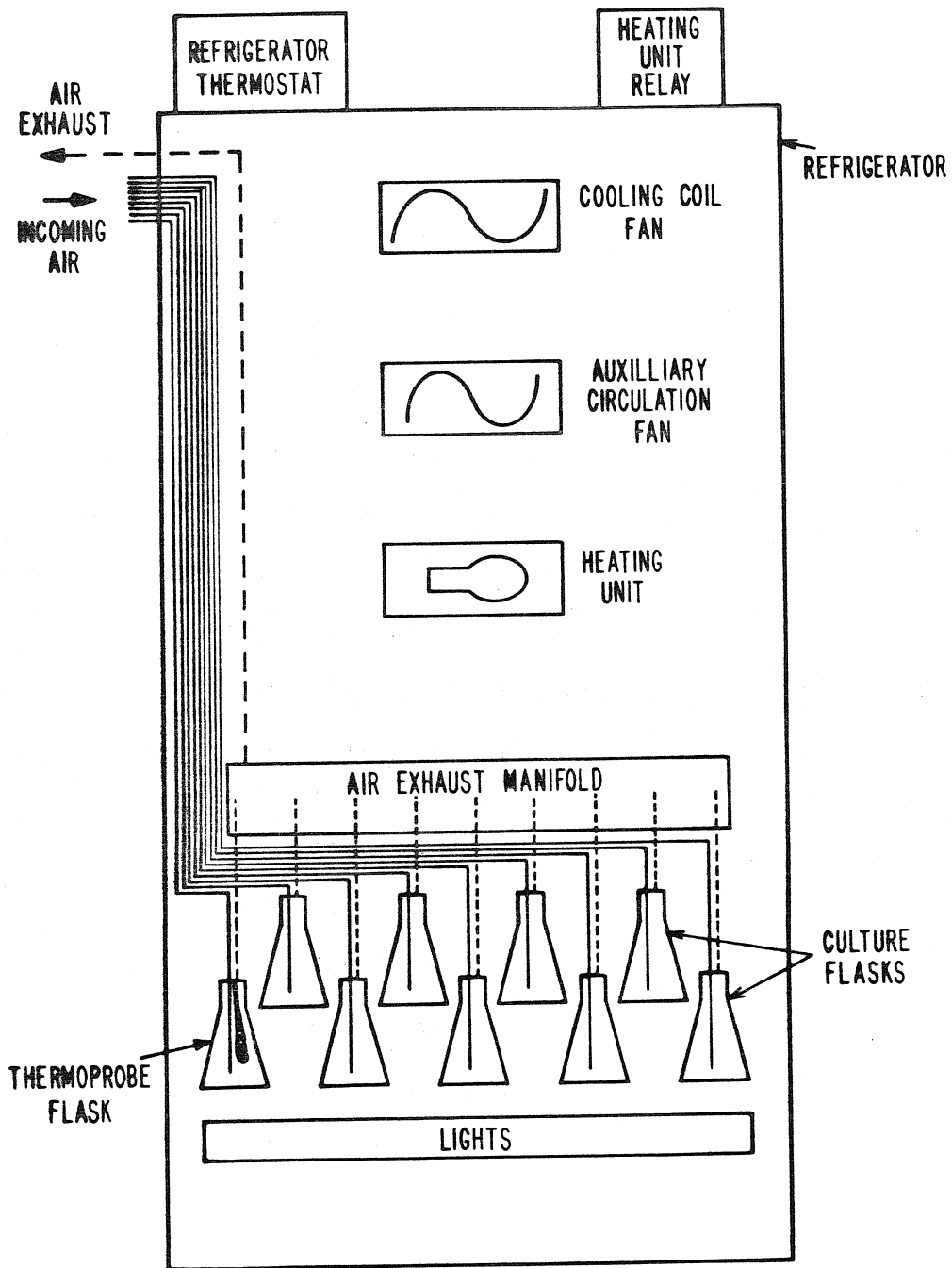


Figure 2.3 Schematic of growth chamber used to culture Macrocyctis in cleanroom experiments.

GE Gro'nSho fluorescent lights sealed in all-plastic fixtures. Illumination was  $1.6 \times 10^4$  ergs per  $\text{cm}^2$ -sec which saturates photosynthesis in Macrocystis (Clendenning 1971). Filtered air was delivered to each flask individually, and subsequently carried away by tygon tubing.

#### 2.4.3 Temperature Control

An auxiliary fan aided air flow over the cooling coils, and an eight inch feather fan supplemented air circulation throughout the interior chamber (Figure 2.3). A new temperature control was added to the freezer, but proved insufficient. A secondary temperature control system was added, consisting of an intermittent heating cycle to provide fine adjustments to the constant cooling of the chamber.

An electric thermoprobe (Model T260 Magna-Set thermoregulator, Princo Instruments, Inc.) (Figure 2.4) signalled a relay when chamber temperature was below the desired (set) value. The relay then turned on a 75-watt incandescent lightbulb heating unit. Once the temperature rose to the set value, the thermoprobe again signalled the relay, shutting off the heating unit after the set temperature was attained.

#### 2.4.4 Air System

Filtered air was passed through the flasks at one liter per minute to simulate turbulent ocean conditions. Air from the laboratory supply was passed through zeolite (Linde molecular sieve) to remove oils and hydrocarbons, then through a Gellman all-plastic air filter (Model

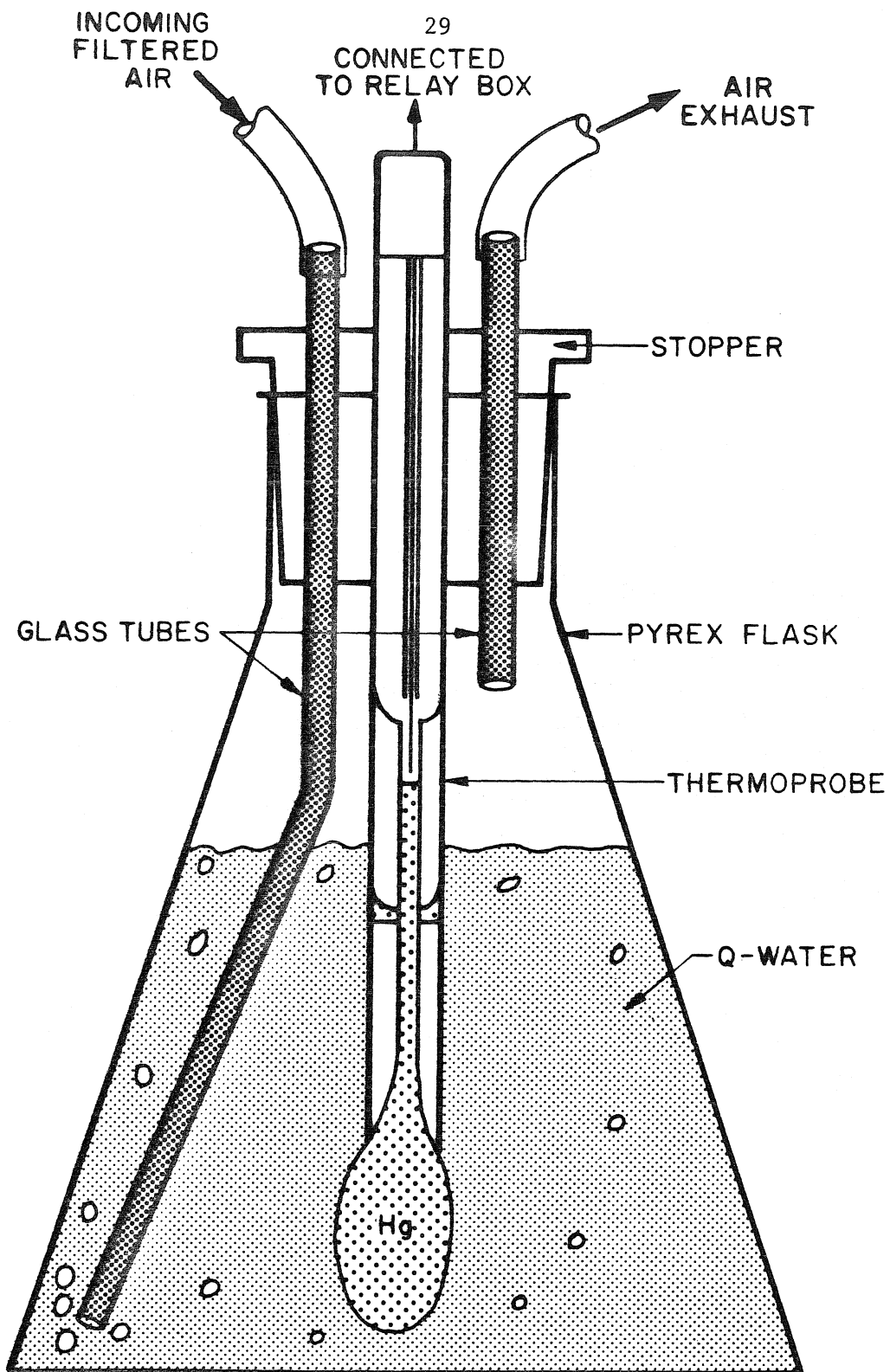


Figure 2.4 Electric thermoprobe apparatus for secondary temperature control of growth chamber.

#12570) to remove particles down to 0.20  $\mu\text{m}$  (Figure 2.5). Filtered air was then metered to each flask by individual glass manometers, calibrated with a glass and teflon Gilmont flowmeter (Model #F1300) before each experiment (Figure 2.6). Tygon tubing with polypropylene tube fittings (Ryan Herco) brought air to each flask through a small opening in the back of the refrigerator. The exhaust air contained salts and was very corrosive. It was exported from the refrigerator in tygon tubing (Figure 2.3) and discharged through the fume hood.

## 2.5 Juvenile Macrocyctis

Macrocyctis pyrifera (Linnaeus) Agardh is a large brown alga inhabiting the California coast as well as temperate waters adjacent to every major land mass in the southern hemisphere. "The life cycle of Macrocyctis involves an alternation of generations between asexual large sporophytes and sexual microscopic gametophytes" (North (1971), Figure 2.7). This research used the early juvenile sporophyte, part of the macroscopic phase of the life cycle. All sporophytes were single blades with stipes and holdfasts (Figure 2.8). Starting weights were 5.0 mg, or about four to seven weeks beyond the microscopic stage.

### 2.5.1 Culturing Test Plants (Figure 2.9)

Scuba divers gathered spore-bearing blades from mature Macrocyctis off Corona del Mar, California. The blades were immediately returned to the Kerckhoff Marine Laboratory for processing. Reproductive blades released their spores after soaking 30 minutes in

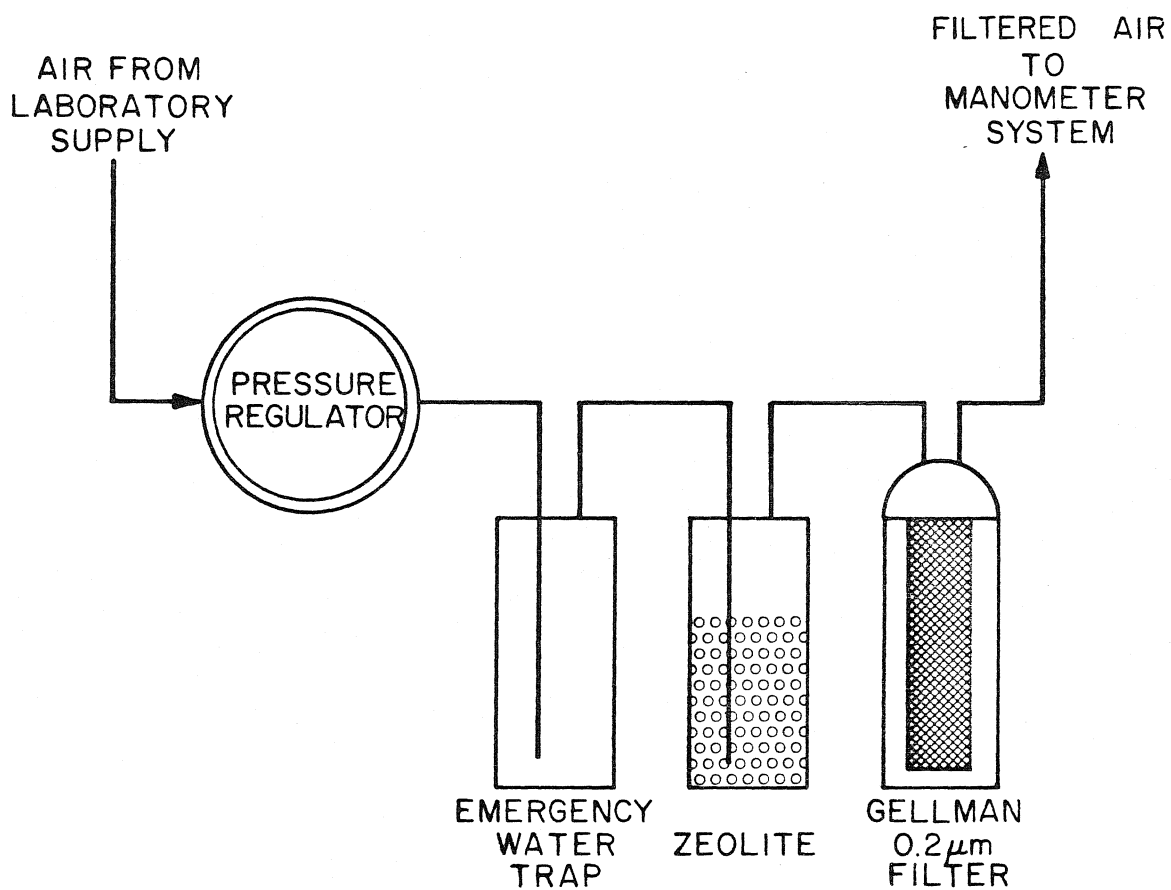


Figure 2.5 Air filtration system for flow to flasks in growth chamber.



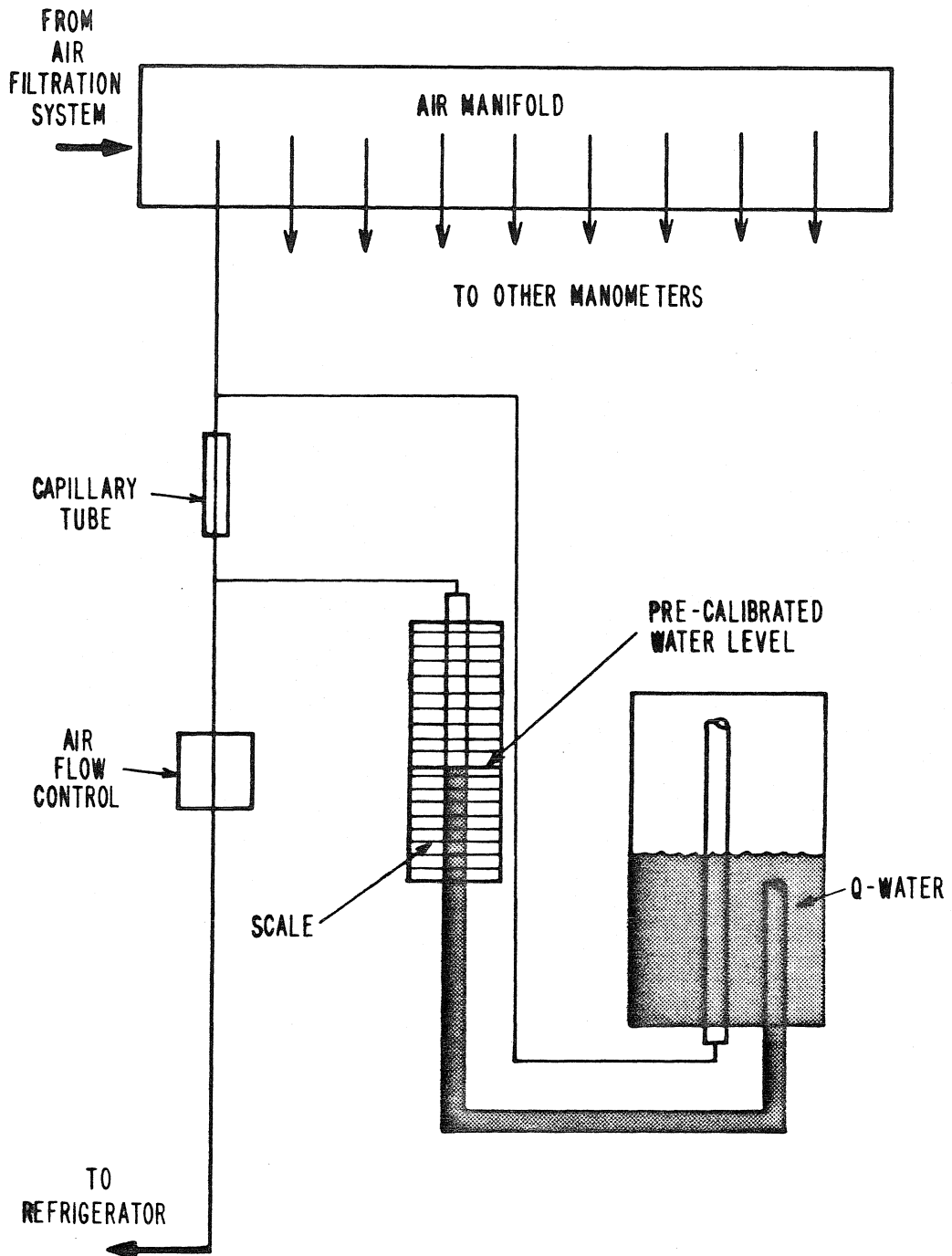


Figure 2.6 Individual air metering system for flasks in growth chamber.

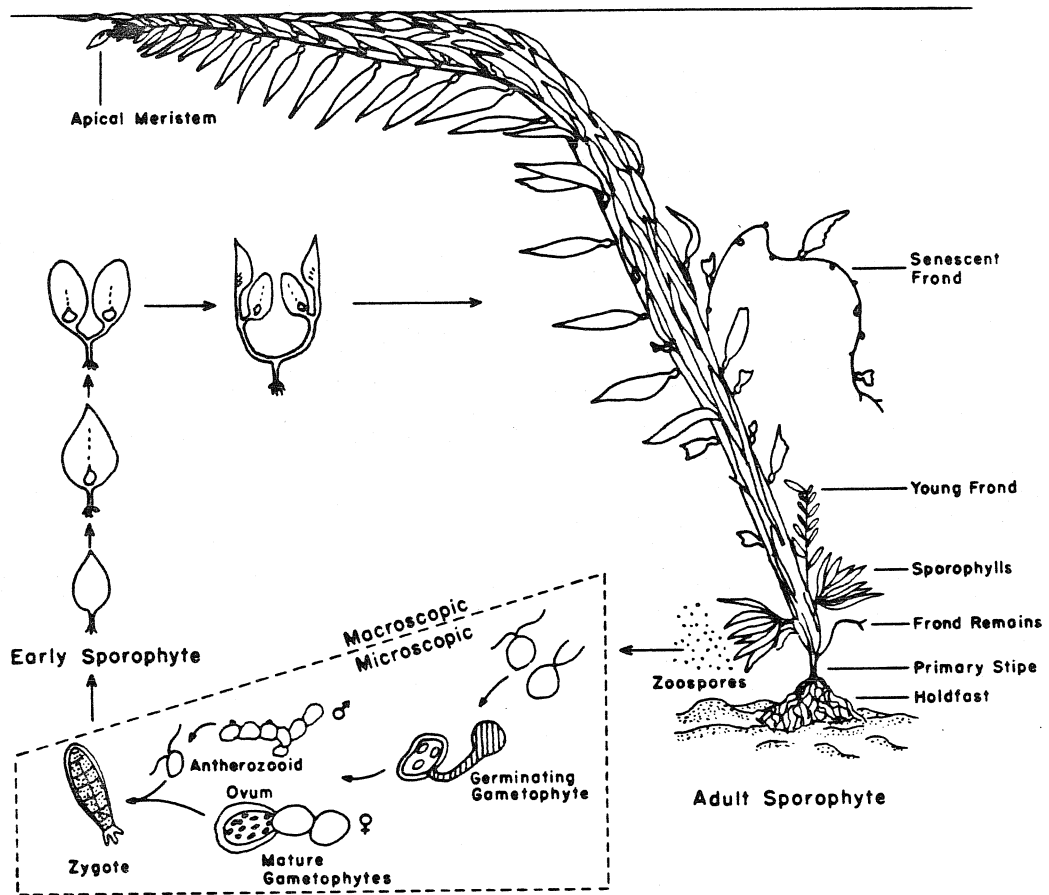


Figure 2.7 Life cycle of *Macrocyctis pyriferus*.

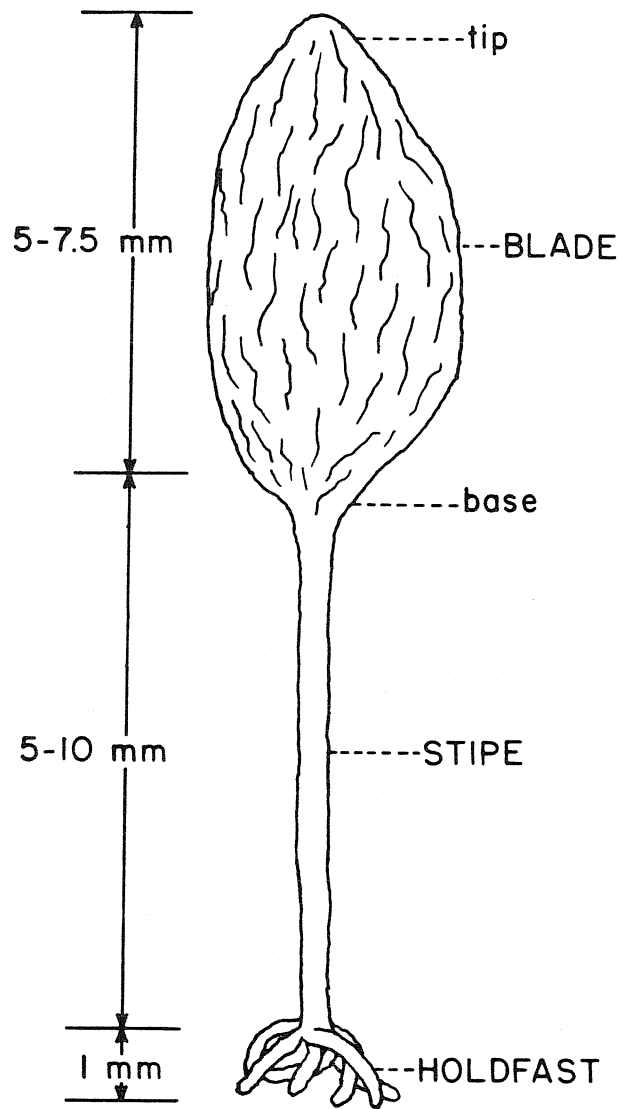


Figure 2.8 Morphology of juvenile *Macrocyctis pyrifera*.

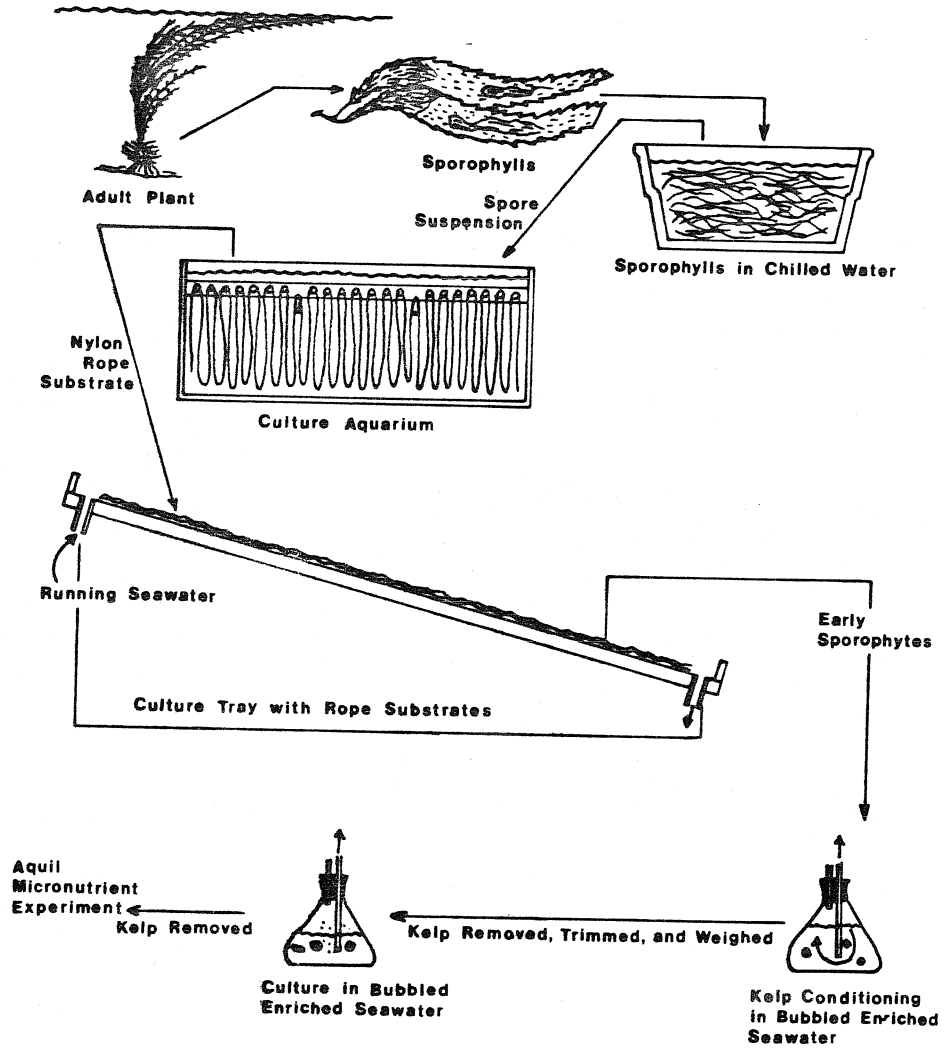


Figure 2.9 Juvenile Macrocyctis culturing and preconditioning.

9°C surface ocean water gathered five miles offshore. The substrate, 1/4" nylon rope, was immersed in the spore suspension for at least 24 hours at 11°C. After several days, ropes were transferred to running chilled seawater from Newport Bay which had been sand filtered and passed through ultraviolet light to eliminate bacteria and other microorganisms. Flow rate in the culturing tray was approximately 50 gallons per minute. Illumination was provided above and below the acrylic tray by 60 watt fluorescent lights.

Juvenile plants were carefully removed manually from the ropes, retaining the stipes and attachment holdfasts. They were transported 54 miles to Keck Laboratory in Pasadena in a flask of surface ocean water embedded in shaved ice. Transportation temperatures remained between 3°C and 10°C.

#### 2.5.2 Condition of Rope-Cultured Plants

Only healthy juvenile plants without any visible signs of disease or nutritional deficiency were chosen from rope cultures for experiments. Newport Bay water failed to sustain rope cultures during certain seasons. Growth was slow, and plants appeared stunted. Development from gametophytes to visible sporophytes required very long periods. Experimentation was halted until growing conditions improved.

Occasional equipment problems such as loss of temperature control adversely affected rope cultures. Damaged rope cultures were not used

until surviving plants exhibited several weeks of healthy growth. This was defined by various physical appearances used to distinguish between healthy and diseased morphology in juvenile kelp tissues (Figure 2.10).

### 2.5.3 Preconditioning

Preconditioning ensured that the recent nutritional and physical histories of all juveniles were identical. Preconditioning presumably reduced plant variability, thereby clarifying any reactions to the various micronutrient levels in the Aquil. Attempts were made to control genetic variability by obtaining microscopic cloned sporophytes from University of California, Santa Barbara. The plants had been grown in a highly concentrated Provasoli medium. They could not be effectively filtered out to replace the medium as long as they were microscopic in size (Virginia Kuga, University of California, Santa Barbara). Time for the microscopic clones to reach 5 mg weight was unpredictable and transfer to low-nutrient Aquil apparently shocked the small plants, causing large variations in growth. These two factors outweighed the possible benefits from reducing variability by use of genetically uniform plants, so they were not used in the experiments.

Juveniles were kept suspended en masse in 2 L of offshore surface seawater by bubbling with ambient air at one liter per minute at the Keck Laboratory cold room and illuminated by 60 watt fluorescent lights. They were conditioned for a minimum of three days until they reached a weight of six to eight mg. The surface ocean water was augmented with 15  $\mu\text{M}$   $\text{NO}_3$  and 1  $\mu\text{M}$   $\text{PO}_4$  and replaced every three days. The

### Healthy



long, narrow



short, broad



curled



folded



smooth



wrinkled

### Unhealthy



paling tip



shot holes



tear



tissue loss



twisted tip



tip at 90°  
(side view)



black algal  
contamination

Figure 2.10 Morphology of healthy, stressed and diseased Macrocystis juveniles.

combination of augmented surface ocean water and Pasadena air almost certainly provided enough micronutrients to satisfy nutritional requirements of the tiny plants.

#### 2.5.4 Variations in Preconditioning

Chemical composition of ocean water used to precondition juvenile plants may have fluctuated slightly but not nearly to the extent of variations in water quality of Newport Bay. Factors causing changes included periods of upwelling and storms accompanied by runoff. Surface ocean water, however, collected five miles offshore was probably more stable than nearshore or harbor water. High metal concentrations of copper and zinc found in harbor water probably reflect commercial inputs to the bay (Young and Alexander, 1978).

#### 2.5.5 Plant Weighings

Juvenile sporophytes were weighed while wet to determine biomass production, which in turn measures suitability of the various Aquil media.

Plants were withdrawn from the flasks for the final weighing with an all-teflon scoop, gently blotted with Scott Microwipes tissues, and immediately weighed. Daily weighings were used in our early experiments because they provided a current account of experiments in progress. Blotting, however, traumatizes the delicate tissues, so daily weighings were discontinued. Weighings every three days, corresponding to media replacements, also proved detrimental to the sporophytes. Finally,



plants were weighed only at the beginning and end of experiments. Minimal contamination arose from handling and exposure to air from only two weighings.

Juveniles in experiments #10 through #30 were weighed on a top-loading digital Sartorius balance (Model #1212MPBCD) with a rapid two-second readout to 0.001 g. Plants from Series #40 through #90 were weighed on a newly available Mettler HL52 digital balance, sensitivity 0.00001 g, because it was free of the cleanroom's equipment vibration.

#### 2.5.6 Preparation of Test Plants

Test plants were carefully chosen weighing six to eight mg wet and as morphologically similar as possible. The stipe and blade tip were removed using acid-cleaned glass cover slips. The plants were gently blotted with Scott Microwipes, weighed immediately, and those exceeding  $5.0 \pm 0.5$  mg were discarded. Acceptable plants were immediately plunged into 250 mL flasks of chilled surface ocean water, three plants per flask. The tiny blades were all handled manually without gloves, because teflon tweezers or plastic gloves bruised the delicate juvenile tissue. It was possible to pick up the juveniles by the stipe manually without gloves, but not with gloved hands or with tweezers.

Plants in the 250 mL flasks were bubbled for one hour and the surface ocean water was then replaced. This procedure removed polysaccharides which oozed from the blade surface for at least an hour

after trimming (North, personal communication). Plants were removed from flasks by pouring contents through an FEP teflon mesh (Tetko, Inc.).

#### 2.5.7 Growth Flask Conditioning

Each 1 L bottle of prepared Aquil contained 950 mL of medium. The growth apparatus was conditioned with 50 mL of prepared Aquil and three test plants. The apparatus was shaken, and the 50 mL Aquil was carefully poured out, retaining the plants. The remaining 900 mL was immediately added. The apparatus was then attached to the air system in the growth chamber.

#### 2.5.8 Growth Parameter

The suitability of various Aquil formulations for the kelp was assessed by computing specific growth rates (Eppley et al., 1969) where

$$R = t^{-1} \ln \left[ \frac{W_f}{W_i} \right]$$

where

- R = specific growth rate, per day
- t = time elapsed, days
- $W_i$  = initial wet weight of the juvenile, g
- $W_f$  = final wet weight of the juvenile, g

Previous laboratory research indicated that juveniles (10 to 50 mg)

can grow maximally at about 0.30 to 0.35 per day for a week. Average rates typically decline each subsequent week, giving values of about 0.25 per day during the second week and 0.20 to 0.23 per day during the third week (North, personal communication). Each size range has a characteristic mean value of R that declines as the plant develops. (North, personal communication.) Direct comparison with other species or large Macrocystis plants is thus not possible. Healthy young Macrocystis juveniles grew an average 0.9 per day in nature (North, 1978c).

Other growth parameters were considered, such as lengths and areas of blades. Maturing blades showed marked variations in length and width, developing ridges and folds without relation to tissue health. Area measurements required flattening blades which might damage tissues. Area-density relationships were also highly variable.

Weight gained by plants directly expressed biomass production as well as an integrated measure of response by blades to the medium, independent of morphology. Biomass increase was of primary concern to the parent project.

Four growth rate categories were established for the size range of plants grown in this study:

R = 0.05-0.15 per day	Poor
R = 0.15-0.25	Fair
R = 0.25-0.35	Excellent
R = 0.35 and over	Exceptional

#### 2.5.9 Temperature Corrections for Specific Growth Rate

The growth chamber was designed to hold the temperature at 10°C throughout the interior. There were temperature variations from flask to flask that were not eliminated until the secondary temperature control (heating) system was incorporated.

The specific growth rate, R, at a temperature T<sub>2</sub> was converted to R' at 10°C (the design temperature), according to Clendenning (1971) by the equation:

$$R' = R [1.7^{(0.1T_2-1)}]^{-1}$$

This conversion was used for experimental Series #20 through #50, and was unnecessary for subsequent experiments.

#### 2.5.10 Criterion of Statistical Significance

Mean growth rates were calculated by grouping all plants from the same Aquil formulation and series. Differences between means were analyzed for significance by t-testing at p = 0.05.

#### 2.5.11 Plant Preparation for XRF

Blotted plants after the final weighing were placed on clean polyethylene sheets, covered with new gauze and a blotter, and pressed between two sheets of cardboard (Figure 2.11). After several weeks of drying, specimens were sent to Crocker Nuclear Laboratory at University of California, Davis, for x-ray fluorescence analysis (XRF).

XRF data provided only total elemental compositions of tissues. Such values resulted from active and passive uptake as well as inert adsorption. Anomalously high values might indicate contamination during experiments.

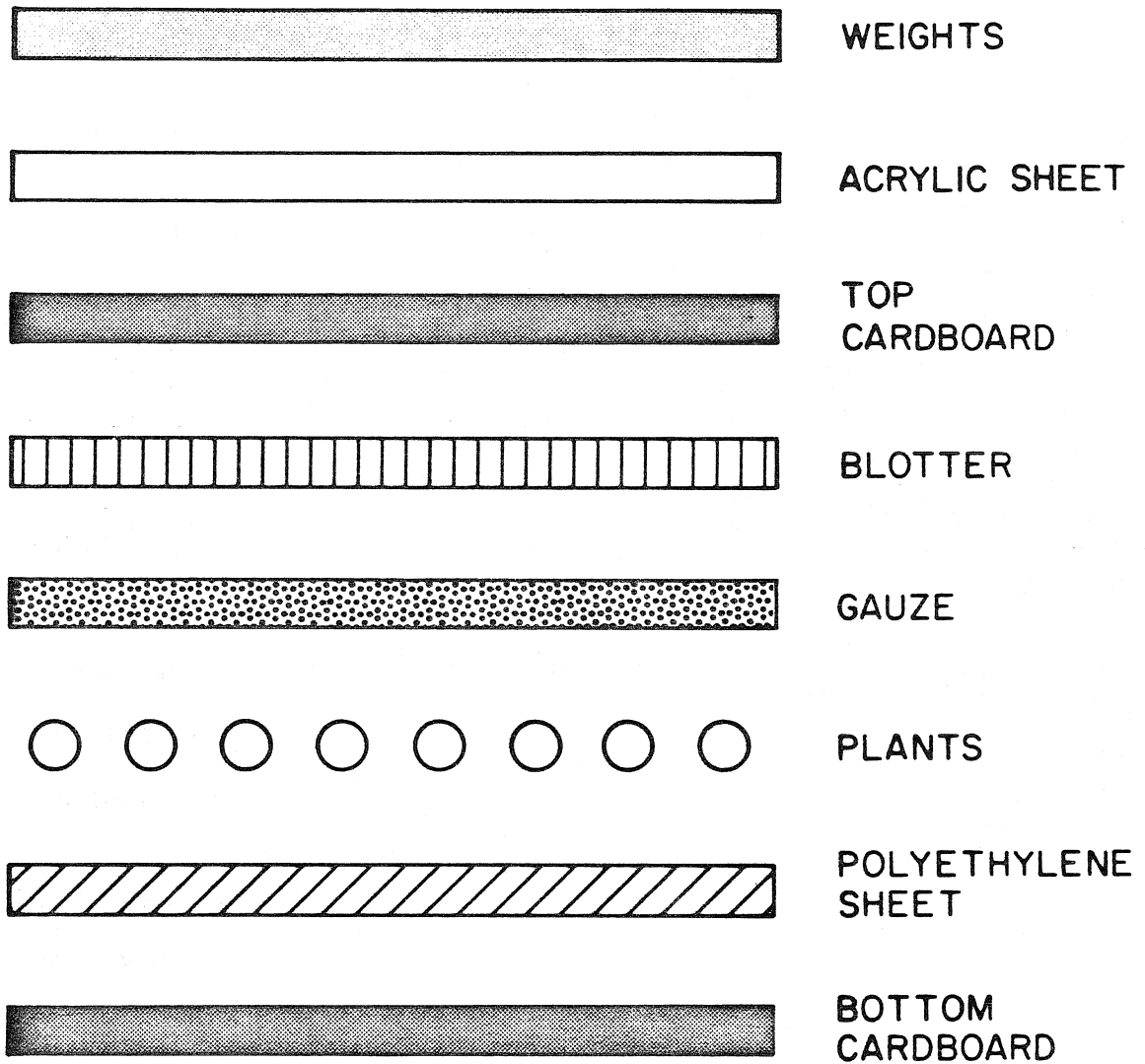


Figure 2.11 Macrocystis tissue preparation for X-ray fluorescence analysis.

## Chapter 3

## IRON REDUCTION BY MACROCYSTIS: CONDITIONS AND RESULTS

3.1 Introduction

Iron occurs in the ocean at low concentrations and is required for metabolic processes of virtually all terrestrial and marine organisms. Iron functions as an electron carrier in oxidation-reduction reactions in cytochromes and ferredoxin. Iron primarily exists in the ferric ( $\text{Fe}^{3+}$ ) state in aerobic marine environments, forming highly insoluble hydroxides and phosphates. A dynamic cycle reducing Fe(III) to Fe(II), and then oxidizing back to Fe(III) through interactions with oxygen and organics has been theorized (Morgan and Stumm, 1964). Total iron ranges from about 10 to 35 nanomoles per liter (nM) in oceanic seawater (Brewer (1975), North (1980), Windom and Wallace (1980)) and probably exists largely as ferric hydroxide,  $\text{Fe}(\text{OH})_3$ .

3.1.1 Hypotheses for Iron Uptake (Figure 3.1)

There are three possible hypotheses for removal of iron from Fe-ligand chelates by organisms in experiments such as described below. One proposes that the complex is reduced and then dissociates at membrane surfaces. The second suggests that chelates are taken up intact and subsequently separated inside the cell. A third possibility is dissociation at the membrane surfaces.

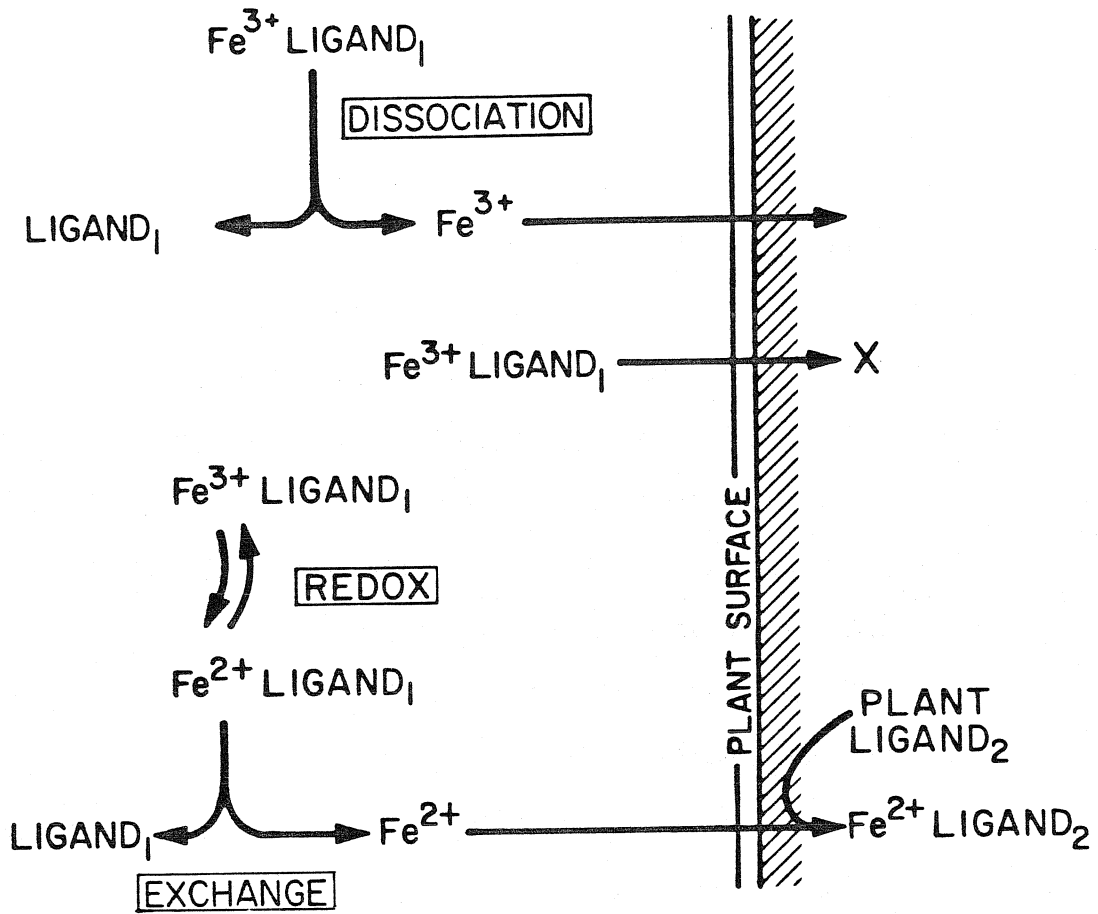


Figure 3.1 Three hypotheses for iron uptake.



### 3.1.2 Previous Research

A few early experiments with soybeans supported the second theory (Jeffreys et al., 1961). Subsequent experiments substantiated the first or non-stressed plants theory, for several terrestrial plants. These experiments showed that non-stressed plants absorbed iron in large amounts but not the ligand. This suggests that the mechanism was reduction of the iron-ligand complex followed by separation (Tiffin et al., 1960). Chaney et al. (1972) found that excess of bathophenanthroline disulfonic acid (BPDS) (a ligand with a strong preference for  $Fe^{2+}$  over  $Fe^{3+}$ ) prevented iron uptake, and  $Fe(II)(BPDS)_3$  accumulated in solution. Owens and Chaney (1971) reached the same conclusion, exposing the fresh water unicellular green alga Chlorella sorokiniana to BPDS. Sufficient  $Fe^{2+}$  added to complex all the BPDS reversed the inhibition. Orthophenanthroline, a ferrous chelator with properties similar to BPDS, partially inhibited growth by the marine diatom, Thalassiosira fluviatilis (Michael Anderson, personal communication). Jackson and Morgan (1978) cited experiments showing EDTA did not cross the phytoplankton cellular membrane, further supporting the dissociative theory.

Bailey and Taub (1980) recently examined effects of hydroxamate siderophores (strong  $Fe(III)$  chelators) on growth of two green algae, Chlamydomonas reinhardtii and Chlorella vulgaris. Siderophores purified from excretions of the blue-green alga Anabaena sp. inhibited Chlamydomonas but not Chlorella. Addition of excess iron overcame the

inhibition. Weaker chelators did not affect growth of either alga. Competition among algae for iron could thus be important under low iron conditions. Krauss and Sprecht (1958) found that the freshwater green alga Scenedesmus broke down  $^{14}\text{C}$  labelled EDTA. This implies that some algae obtain metals by degrading chelates.

Although most evidence indicates that ferrous iron is separated from the chelate, elements crossing plant membrane surfaces may not be free metal ions. Possibly iron releases from the EDTA complex, binding to ligands on surface membranes. The hypothetical ligand could be highly specific for certain metal cations, facilitating transport across cellular membranes. Black and Mitchell (1952) concluded that many trace metals occur as insoluble forms and may combine with polysaccharides such as alginic acid or fucoidan on surfaces of the brown alga Laminaria. Both polysaccharides occur in cell walls and intercellular spaces of all brown algae. Haug and Smidsrød (1967) suggested that magnesium is accumulated by brown algae via ion-exchange between seawater and plant alginates. Magnesium concentrations are lower in algae than in seawater, but specificity of the polysaccharides for cations such as iron or manganese could aid in active transport. No information exists on iron-binding properties of cell membrane surfaces.

Iron may also be released from chelates to plant excretions. Organisms enhance uptake by a variety of mechanisms. Lichens solubilize metals by liberating acids (Schatz, 1963). Desferrichrome binds and carries iron into cells after extraction from alum shales by fungi

(Napier and Wakerly, 1969). Jackson and Morgan (1978) compared reported observations of phytoplankton growth in seawater media with theoretical analyses using diffusion, dissociation transport and iron exchange in the Fe-EDTA complex. They were unable to account for phytoplankton growth enhancement by synthetic chelators and could not rule out iron or manganese limitation of growth.

Photoreduction is an alternative pathway for degradation of the Fe-EDTA complex. Lockhart and Blakely (1975) found photoreduction of the Fe-EDTA complex decreased with increasing pH. Fe(III)EDTA completely photodegraded at pH 8.5 after 32 hours of illumination by 4000 foot-candles. This is approximately ten times the saturation level for Macrocystis (Clendenning, 1971).

### 3.2 Experimental Conditions

#### 3.2.1 Laboratory Culturing

Juvenile kelp were grown under several conditions to test whether Macrocystis juveniles reduce Fe(III)EDTA external to the blade (Figure 3.2). Flasks of Aquil were tested under illuminated and dark conditions, with and without kelp, and with and without addition of the strong Fe(II) chelator, BPDS (Table 3.1).

Macrocystis juveniles came from rope cultures as described in Chapter 2. Plants were conditioned in surface ocean water enriched with 15  $\mu\text{M}$   $\text{NO}_3^-$  and 1  $\mu\text{M}$   $\text{PO}_4^{3-}$  for at least 3 days. High

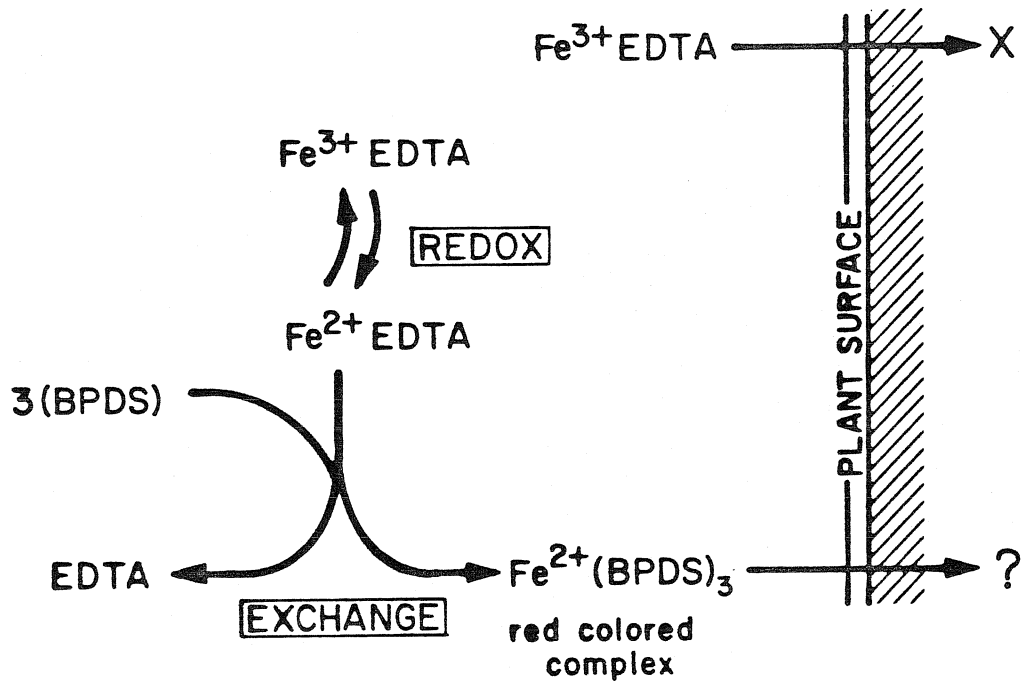


Figure 3.2 Experimental hypothesis for iron reduction prior to uptake.

Table 3.1

Experimental conditions: eight combinations of the variables

	ILLUMINATION		DARKNESS	
	KELP	NO KELP	KELP	NO KELP
BPDS	A <sub>1</sub>	A <sub>2</sub>	A <sub>3</sub>	A <sub>4</sub>
NO BPDS	A <sub>5</sub>	A <sub>6</sub>	A <sub>7</sub>	A <sub>8</sub>

biomass densities were required to cause sufficiently rapid buildup of the red-colored complex  $\text{Fe(II)(BPDS)}_3$  in the media. The high biomass loading of 5 g tissue per 500 mL of Aquil 9 (1000 nM Fe) with 5  $\mu\text{M}$  BPDS was used. Initial juvenile plant weights ranged from 70 to 350 mg. (All other trace metals involved an initial loading of 15 mg per flask.) Experiments lasted a maximum of seven days without replacing media. This allowed accumulation of  $\text{Fe(II)(BPDS)}_3$  so that absorbance rose into the optimal measurement range. Flasks were placed in the growth apparatus as described in Chapter 2, bubbled with air at one liter per minute and illuminated by  $1.6 \times 10^4$  ergs/cm<sup>2</sup>-sec.

### 3.2.2 Absorption Measurement

25-mL aliquots were withdrawn daily from each flask into 30-mL acid-cleaned FEP bottles, analyzed for  $\text{Fe(II)(BPDS)}_3$ , then returned to the flask. Spectra were measured from 200-800 nm on a Hewlett-Packard HP8450A UV/VIS spectrophotometer to measure  $\text{Fe(II)(BPDS)}_3$  concentration. Data were stored on tape cassettes for later analysis and plotting.

$\text{Fe(II)(BPDS)}_3$  has a peak absorbance at 535 nm, (Diehl et al., 1965). Average absorbance over 534-536 nm was computed because the spectrophotometer's measurement interval was 2 nm. A matched pair of quartz cuvettes, 10 cm path length, was used to enhance low-level detection of the  $\text{Fe(II)(BPDS)}_3$ . The cuvette was cleaned between samples by rinsing once with 4 N nitric acid, five times with Q-water and once with a small amount (approximately 1 mL) of the next sample.

Nitric acid was chosen for apparatus cleaning after each use as discussed in Chapter 2. Growth flasks and sample bottles used in BPDS tests in darkness were wrapped with foil and covered with polyethylene to protect media from illumination.

### 3.2.3 Reference Solution

The UV/VIS spectrophotometer measured the absorbance of Aquil in relation to a reference solution. Aquil SB would have been the ideal reference solution because it was the basis for the experimental formulations, but gradual photoreduction and possible biological contamination could have changed absorption of Aquil. Q-water was chosen as the reference solution because biological and abiological changes would not occur, and there were no significant differences in absorbance over the wavelength range of 400 to 800 nm between Q-water and Aquil SB.

### 3.2.4 Standards

Five standards were analyzed to provide a basis for calculating concentration of  $\text{Fe(II)(BPDS)}_3$  from absorbance measurements.  $\text{Fe(II)(BPDS)}_3$  was added to Aquil SB containing 1000 nM  $\text{Fe(III)}$ , at concentrations of 200, 400, 600, 800 and 1000 nM. Spectra of the standards (Figure 3.3) yielded the calculated absorbance vs. concentration curve for  $\text{Fe(II)(BPDS)}_3$  (Figure 3.4).

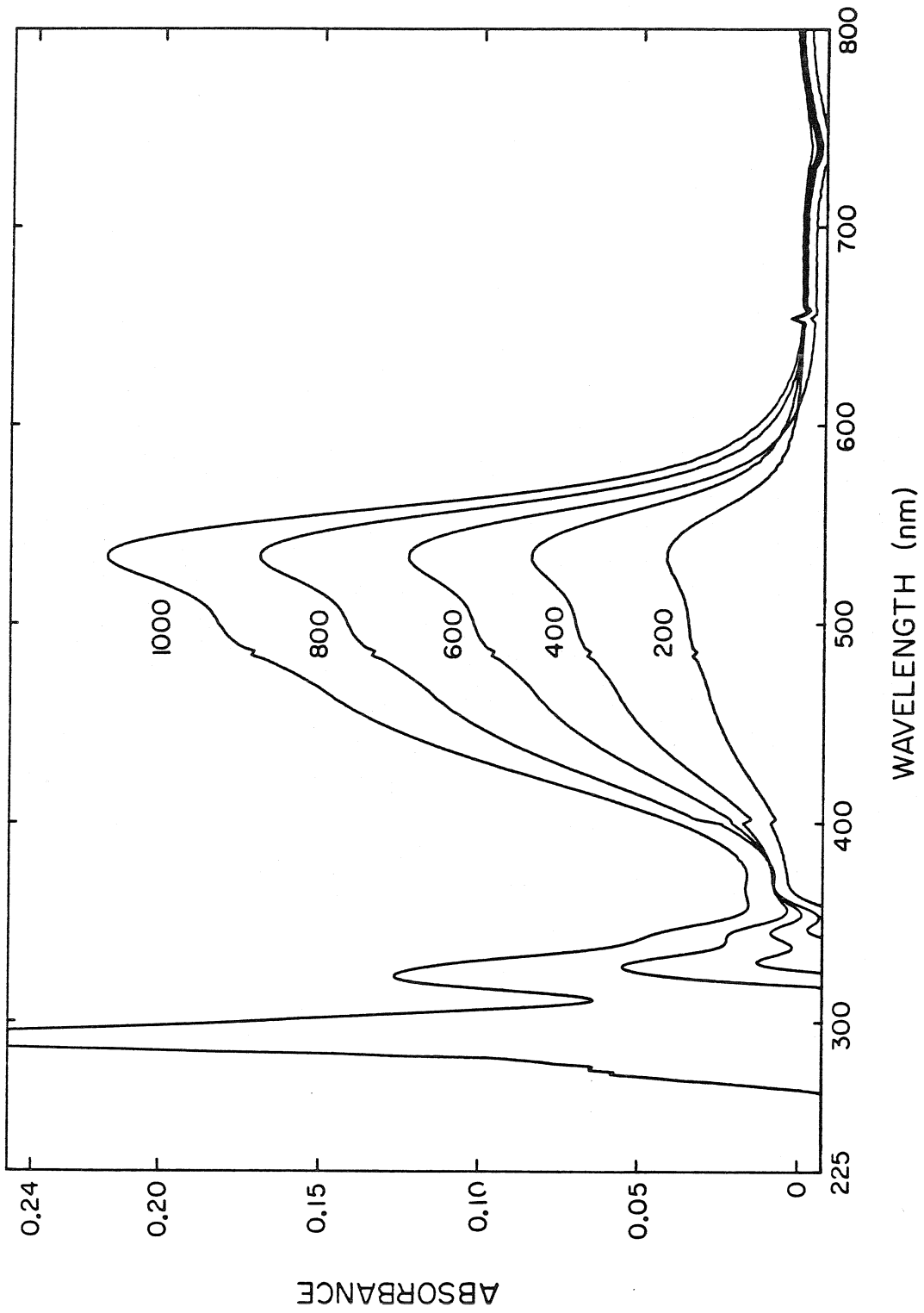


Figure 3.3 Spectra of Fe(II)BPDS<sub>3</sub> standards at concentrations of 200, 400, 600, 800 and 1000 nM.



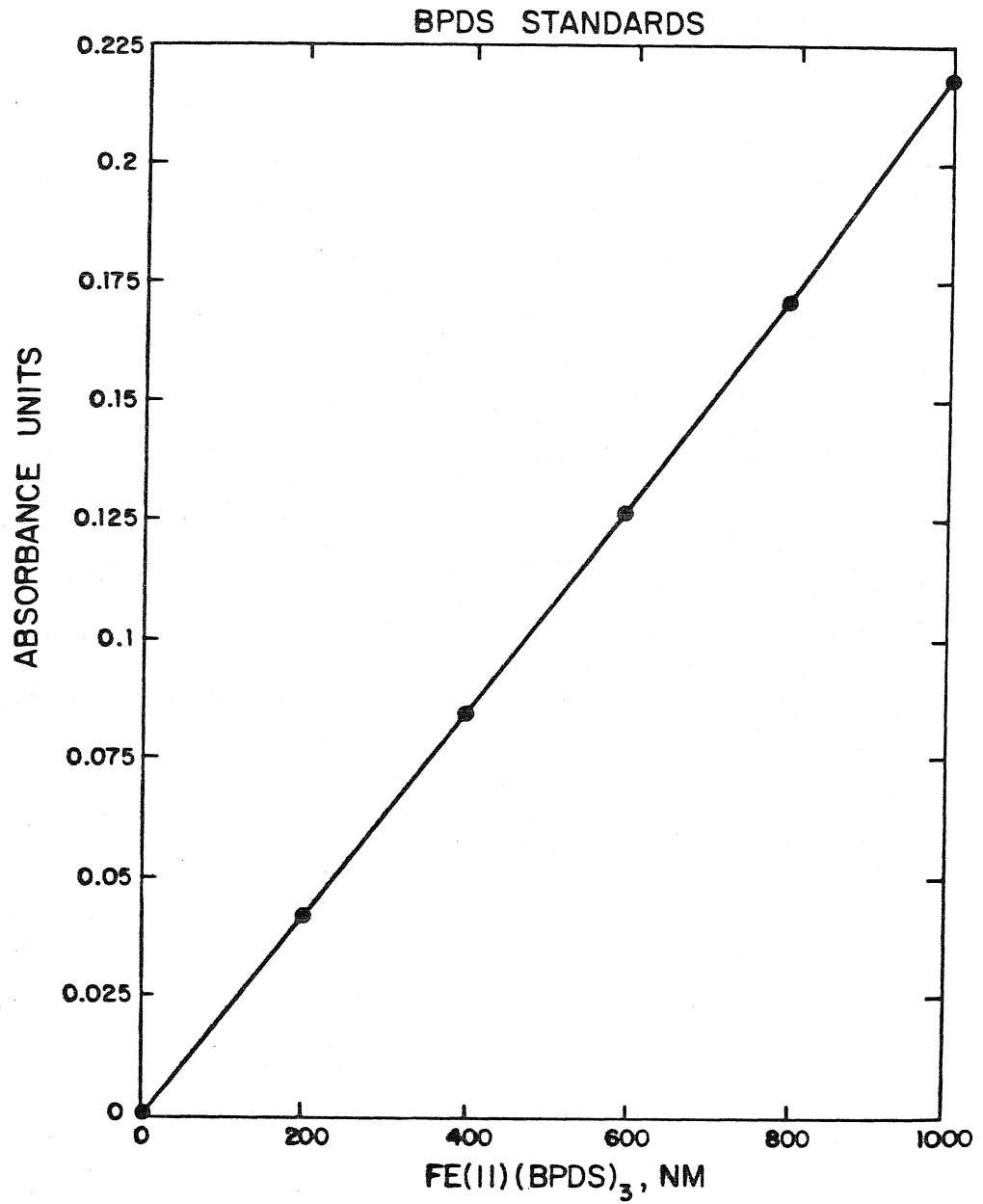


Figure 3.4 Concentration vs. absorbance curve for Fe(II)BPDS<sub>3</sub> standards.

### 3.3 Calculation of Fe(II)(BPDS)<sub>3</sub> Concentration

Baseline was defined as that section of the absorbance curve which characteristically shows no contributions from the Fe(II) BPDS<sub>3</sub> peak. Determination of actual absorbance averaged over wavelengths 534-536 nm required subtracting the average baseline value. Average absorbance over the wavelengths 780 to 800 nm served as a baseline. The absorbance,  $A$ , for a particular set of conditions can be written;

$$A_n(x,y,z) = A_p - A_b$$

where

$A_p$  is the peak average absorbance over 534-536 nm

$A_b$  is the baseline absorbance averaged over 780-800nm

$A_n(x,y,z)$  is calculated peak absorbance for the  $n^{\text{th}}$  set of conditions corresponding to presence or absence of illumination (x), kelp (y) and BPDS (z)

The eight combinations of these variables were labelled  $A_1$  through  $A_8$  (Table 3.1). Five of these showed no significant Fe(II)BPDS<sub>3</sub> peaks: Conditions  $A_4$ ,  $A_5$ ,  $A_6$ ,  $A_7$ , and  $A_8$ . Conditions  $A_4$ ,  $A_6$  and  $A_8$  had zero baselines, while  $A_5$  and  $A_7$  showed non-zero baselines due to presence of kelp.  $A_5$  through  $A_8$  contained no BPDS and absence of peaks ruled out existence of any background artifact interfering with the Fe(II)(BPDS)<sub>3</sub> peak, as well as possible BPDS contamination.

Absorbance curves maintained the same relative positions throughout the experiments (Figure 3.5). Absorption increased daily in the presence of light and BPDS without kelp (Figure 3.6).

Data sets were paired on a day by day basis to separate biological and non-biological sources of  $\text{Fe(II)(BPDS)}_3$ . For example, absorbances for biological activity in the dark were calculated by subtracting any non-biological changes in the same medium:

$$A_3 \text{ (dark, kelp, BPDS)} - A_7 \text{ (dark, no kelp, BPDS)} \quad (1)$$

Similarly, the combined effects of kelp plus light (Figure 3.7) was calculated as:

$$A_1 \text{ (light, kelp, BPDS)} - A_5 \text{ (light, kelp, no BPDS)} \quad (2)$$

Superposition of absorbance curves from sequential days illustrated progressive changes in an experiment (Figure 3.8). The effect of light only (photoreduction) on Aquil was:

$$A_2 \text{ (light, no kelp, BPDS)} - A_6 \text{ (light, no kelp, no BPDS)} \quad (3)$$

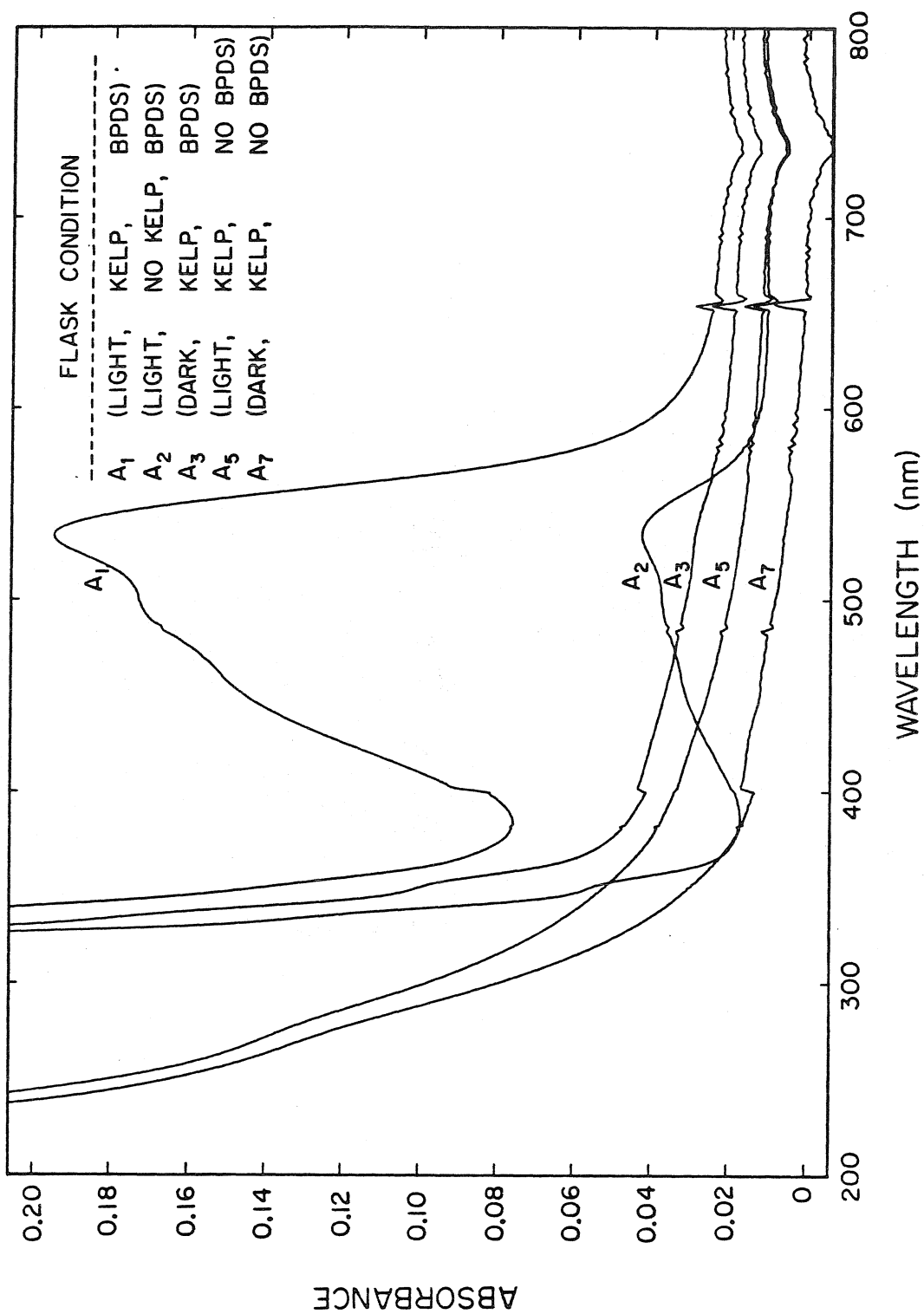


Figure 3.5 Relative positions of the spectra of five experimental conditions.

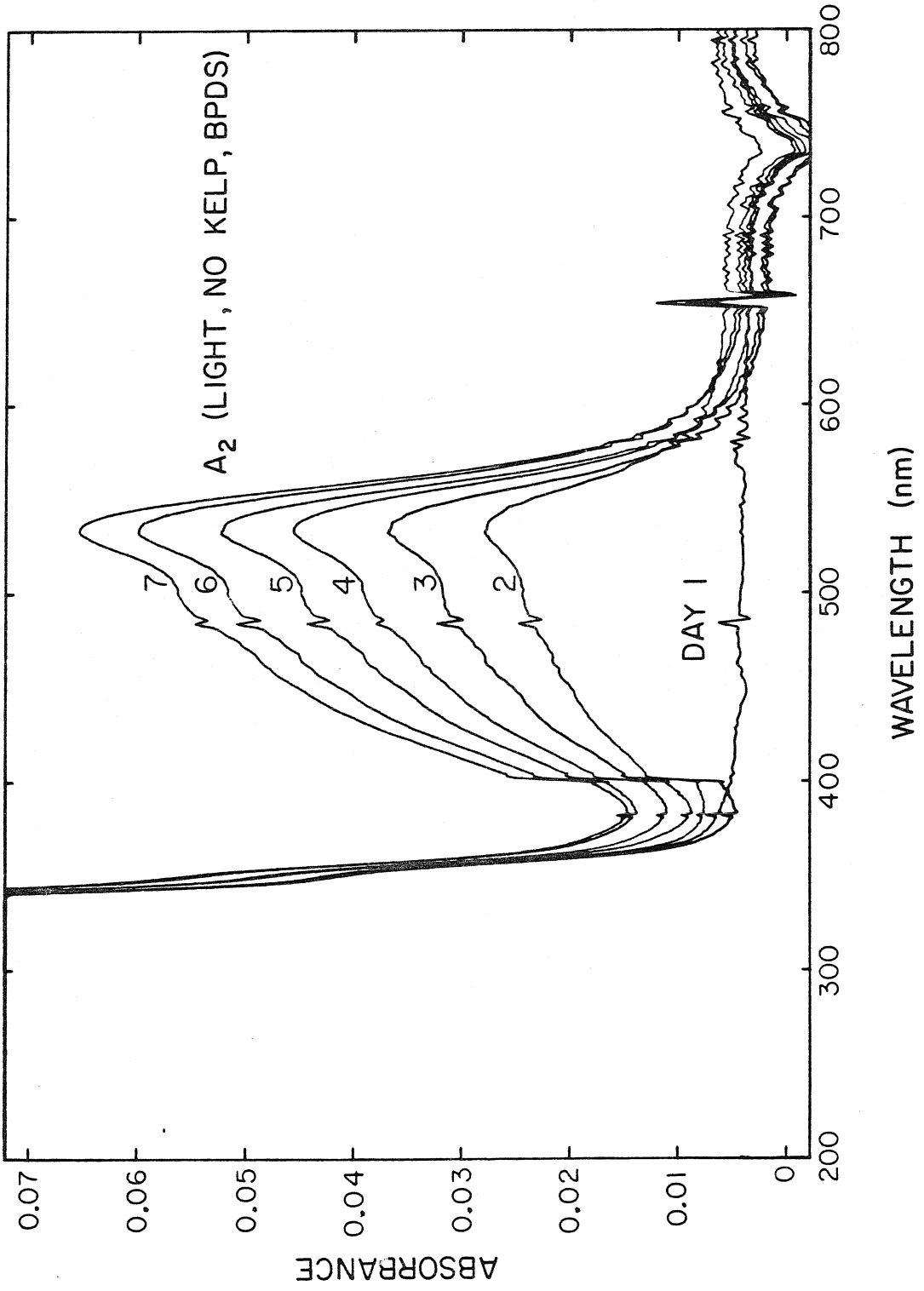


Figure 3.6 Daily absorption increase over seven days for condition A<sub>2</sub>.

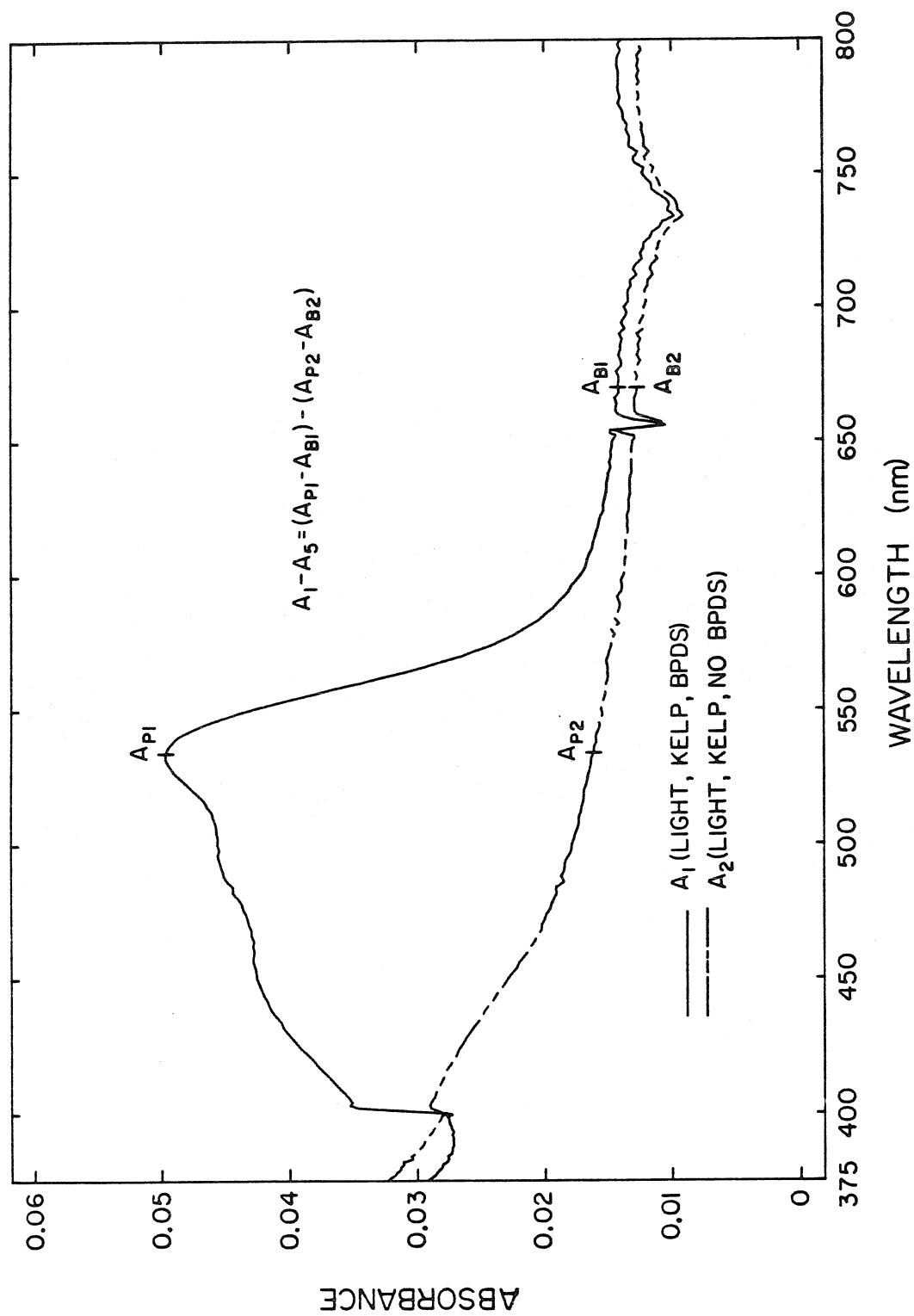


Figure 3.7 Calculation of combined effects of kelp and illumination on Fe(II)BPDS<sub>3</sub> generation.

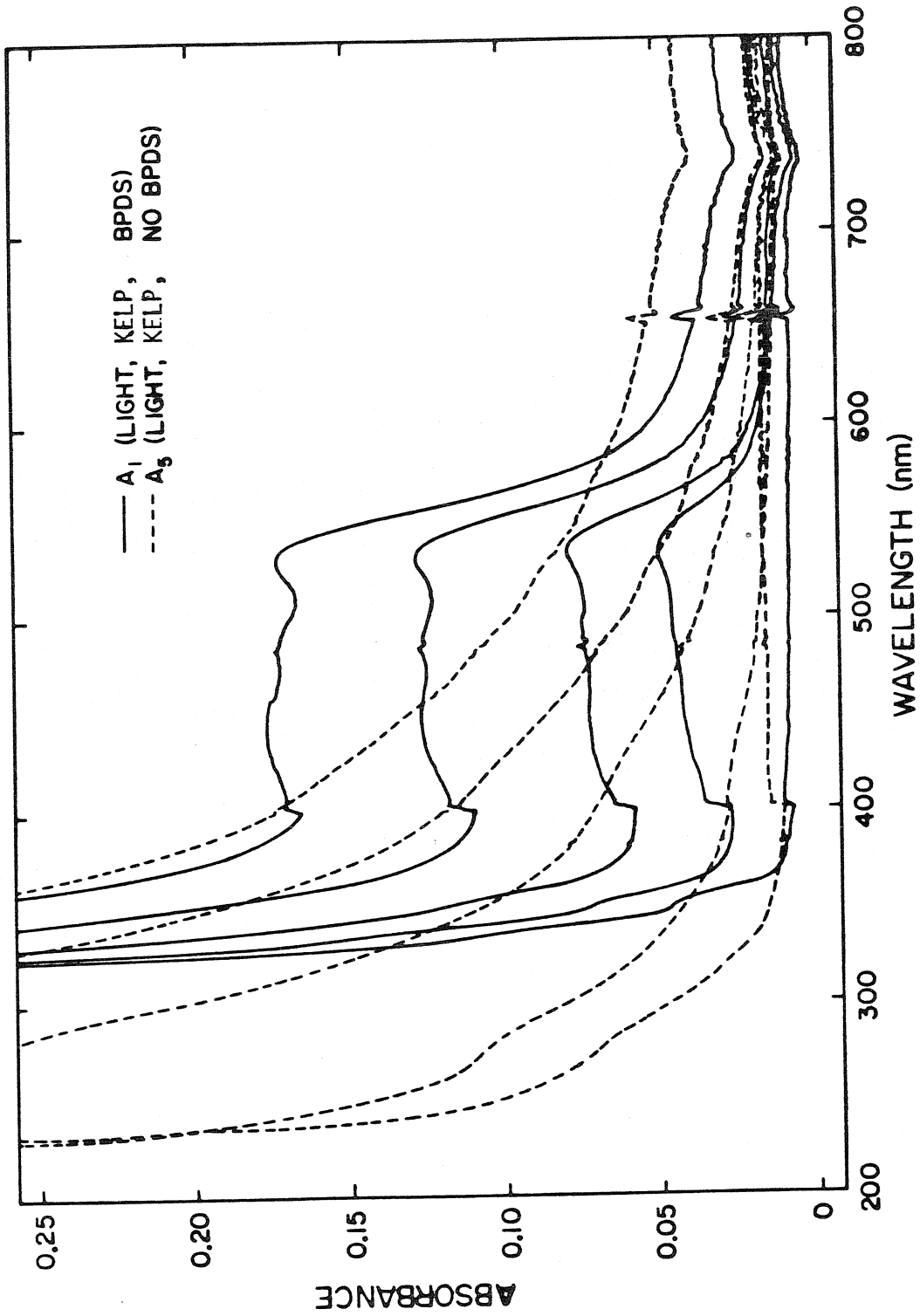


Figure 3.8 Superposition of paired absorbance curves  $A_1$  and  $A_5$  for five sequential days.

### 3.4 Calculation of Results

#### 3.4.1 Eight Experimental Conditions

Three conditions showed significant  $\text{Fe(II)(BPDS)}_3$  production:  $A_1$  (light, kelp, BPDS),  $A_2$  (light, no kelp, BPDS), and  $A_3$  (dark, kelp, BPDS). Conditions  $A_1$ ,  $A_2$ , and  $A_3$  also had non-zero baselines.

The ultimate data combination yielded effects of kelp alone on  $\text{Fe(II)BPDS}_3$  levels present and excluded any photo-induced effects. Kelp-only effects were calculated by subtracting light effects from the combined kelp-plus-light effects:

$$\begin{aligned} & [A_1(\text{light, kelp, BPDS}) - A_5(\text{light, kelp, no BPDS})] \\ & - [A_2(\text{light, no kelp, BPDS}) - A_6(\text{light, no kelp, no BPDS})] \end{aligned} \quad (4)$$

Concentrations of  $\text{Fe(II)(BPDS)}_3$  were calculated for each day from the standards curve. The concentration versus time plots corresponding to Equations 3.2 through 3.4 were combined onto one graph for comparison (Figure 3.9). The most rapid generation of the  $\text{Fe(II)BPDS}_3$  from the combined effects of light and kelp, followed in descending order by kelp effects alone and light effects alone.

The smallest amount of the complex production came from kelp in the dark. A combination of kelp and light reduced 500 nM, or 50% of the initial  $\text{Fe(III)}$  concentration of 1000 nM in five days. Kelp alone



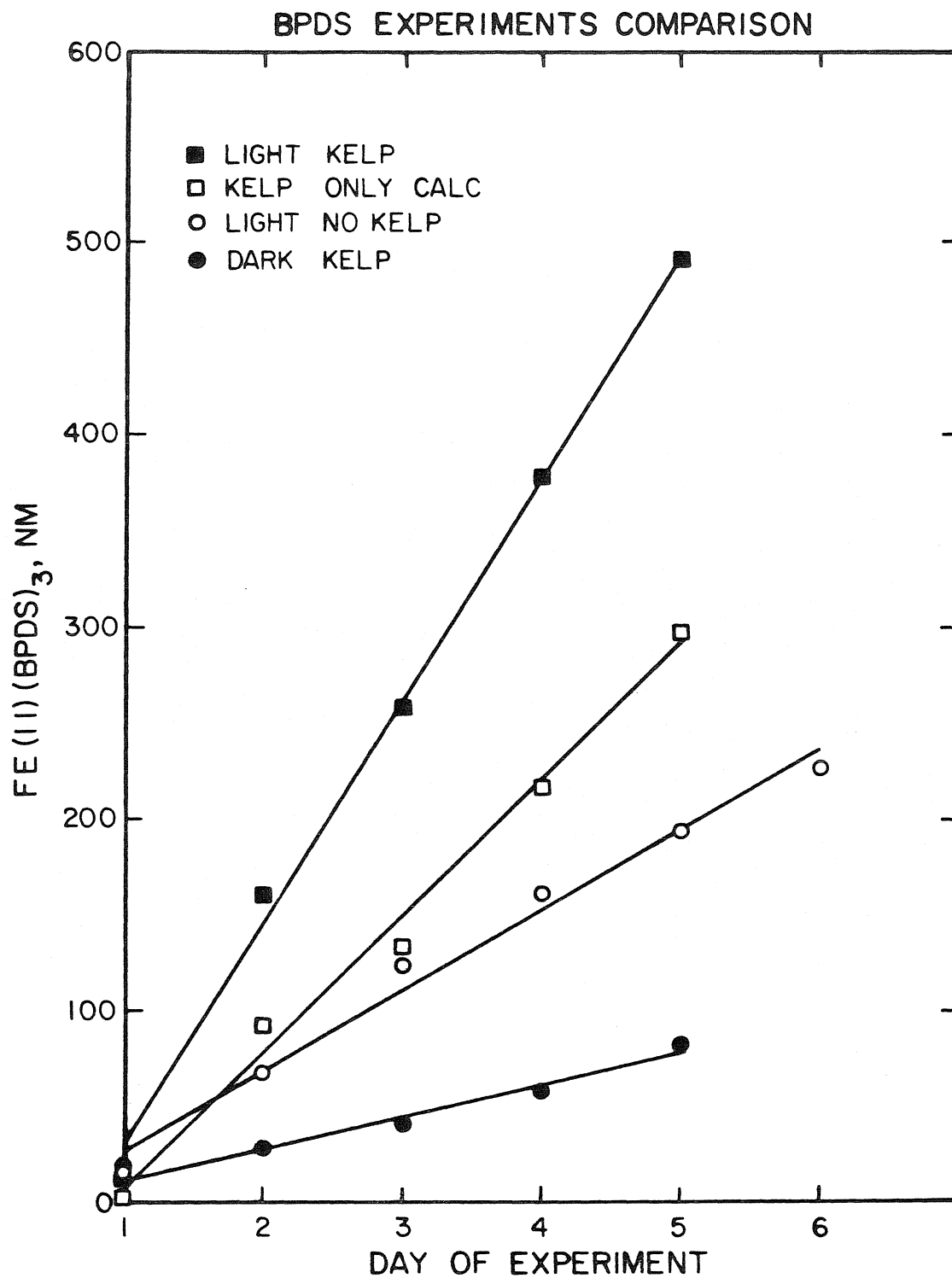


Figure 3.9 Concentration vs. time curves for Fe(II)BPDS<sub>3</sub> generation.

accounted for a 300 nM Fe(III) reduction and light alone for 200 nM. Kelp in the dark reduced iron at a much lower rate than its counterparts in the light, only 83 nM in five days.

#### 3.4.2 Rate of Iron Reduction per Surface Area

Plant areas were determined on the last day of the experiment to permit calculation of rates of Fe(III) reduction per unit area of plant tissue. Outlines of the juvenile plants were traced at the end of the experiment. The tracings were cut out and areas determined by a Lambda Instruments LI-COR Model 3100 area meter. On the last day of the experiment, when changes in tissue area were small, juvenile sporophytes of Macrocystis pyrifera reduced  $9.14 \times 10^{-11}$  moles of Fe (III) per  $\text{cm}^2$  per day.

### 3.5 Summary of Results

1. Juvenile kelp mediated the reduction of Fe(III) to Fe(II).
2. Kelp reduction of Fe(III) was enhanced by light.
3. Photoreduction contributed to reduction rates.
4. The rate of Fe(III) reduction per unit surface area for juvenile Macrocystis pyrifera was  $9.14 \times 10^{-11}$  moles of Fe(III) per  $\text{cm}^2$  per day.

## Chapter 4

## MICRONUTRIENTS EXPERIMENTS: CONDITIONS AND RESULTS

4.1 Introduction

Juvenile Macrocystis were grown in various formulations of artificial seawater Aquil. Plants were cultured, preconditioned, and tested as described in Chapter 2. Before- and after-weighings determined suitabilities of the media. Seven micronutrients were examined; cobalt, copper, iodide, iron, manganese, molybdenum and zinc. A separate series additionally tested Aquil formulations deficient in each of the seven elements.

4.2 Initial Experiments4.2.1 Exceptional Growth Rates

Each experiment used a maximum of eight one-liter flasks, containing 900 mL of Aquil. Initial experiments encompassed 16 to 21 days using juveniles ranging from 5 to 12 mg and 18 to 22 mg wet weight. Media were changed every three days and plants weighed daily. Individual morphology was used to distinguish between each plant in a flask and monitor its daily progress. Daily growth rates, as described in Chapter 2, Section 2.5.8, ranged from a poor 0.06 per day to an exceptional 0.38 per day. Plants of these sizes and growth rates were able to deplete Aquil nutrients before the three-day replacement

occurred.

Trimming during mid-experiment was attempted as a solution to nutrient depletion resulting from exceptionally high growth. Plants were trimmed by 50% on day ten when juveniles weighed from 60 to 120 mg. Plants were returned to the old Aquil and bubbled for an hour to remove the polysaccharides exuded from cut tissues. The medium was replaced and the experiment continued. Trimming did not stabilize growth rates as some slowed while others increased during the ensuing nine days. Two alternative measures were taken to avoid nutrient depletion in subsequent experiments. First, initial weights of experimental plants were reduced to 4.5 to 5.5 mg. These were the minimum weights that permitted handling without damage. Second, experiments were shortened to ten days so that large plants, capable of rapidly depleting the nutrients, never developed.

#### 4.2.2 Synchronized Growth Pattern

Synchronizing growth patterns among three plants in each flask was necessary for comparisons between plants growing in flasks of differing Aquil media. Eight flasks of 4.5 to 5.5 mg plants were grown for 16 days with plant weighings and media replacement every three days. It was found that plants in a particular flask followed synchronized growth patterns as the culturing techniques improved (i.e., growth rates for all three plants increased or decreased in unison over each three day interval as shown in Figure 4.1).

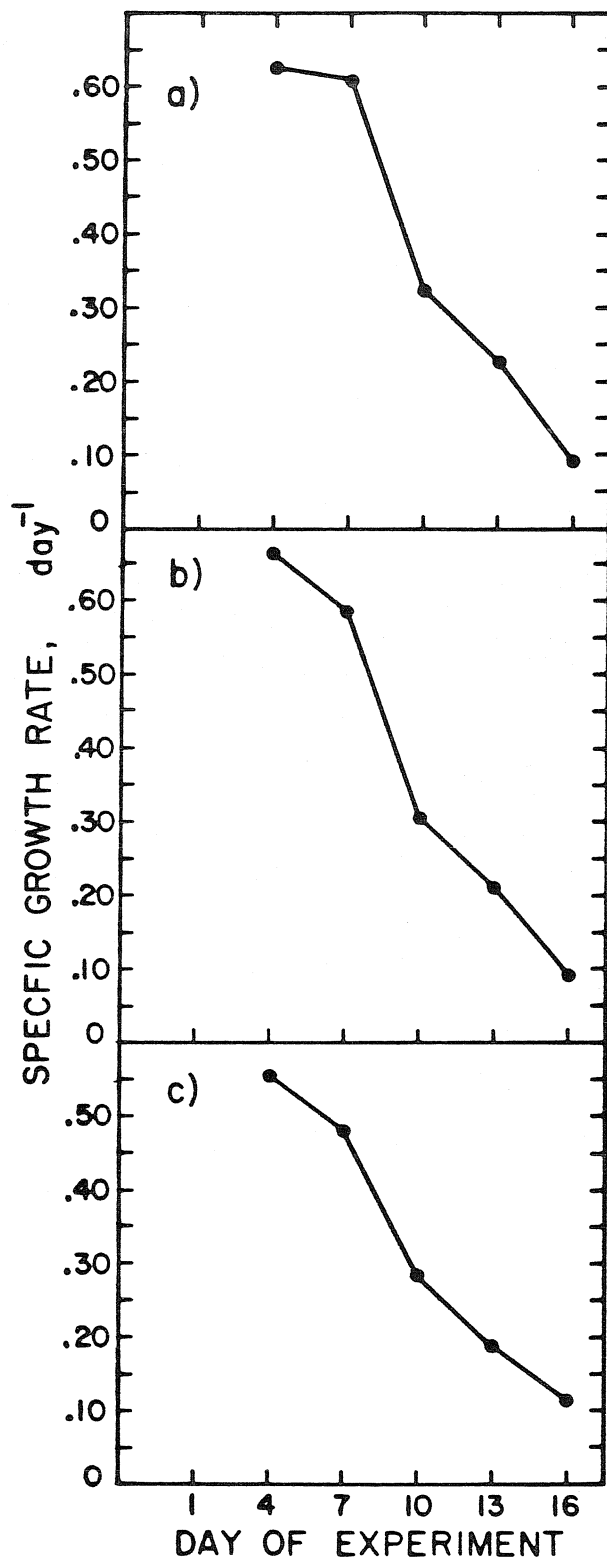


Figure 4.1 Synchronous growth rates exhibited by three plants a), b) and c) grown for 16 days in Aquil SB with 500 nM Fe, 25 nM Mn, 5 nM Cu and 150 nM Zn. Specific growth rates increased and decreased in unison on a daily basis.

#### 4.2.3 Quartz vs. Pyrex Experiment, Series #10

Series #10 tested effects on plant growth from possible leaching of metallic structural components which are more abundant in pyrex than quartz. Four quartz flasks and bubblers were tested against four pyrex flasks and bubblers.

Plants were cultured for 12 days, weighed daily, and media were changed every three days. All eight flasks contained Aquil SB except for 100nM Fe (instead of 250 nM) and 30  $\mu\text{M NO}_3^-$  (instead of 15  $\mu\text{M}$ ). Aquil SB contained standard background levels of micronutrients as described in Chapter 2, and shown in Table 2.1. There was no significant difference between means of growth rates from juveniles in quartz and those in pyrex.

#### 4.2.4 Comparison of Series

Growth rate analyses involved only within-series comparisons. It was discovered that preconditioning could not completely overcome differences in genetic backgrounds and in chemical and physical histories. Such variables presumably caused physiological differences between groups of plants used in our various series. Thus it was decided not to compare growth rates from plants tested in a particular series to rates from plants cultured in the same type Aquil, but in a different series.

A given Aquil formulation was usually tested with two flasks (six plants) within each series. Testing two flasks (6 plants) instead of

one flask (three plants) provided a larger sample size for applying statistical analysis, but only four different concentrations of a particular micronutrient could be used per series because maximum capacity of the growth chamber was eight flasks.

#### 4.3 Scanning Electron Microscopy Examination

A field specimen of kelp tissue was examined by scanning electron microscopy (SEM). Only one sample out of forty survived the difficult "fixing" procedure. SEM pictures showed branching filamentous microorganisms identified as bacteria by Dr. Andrew Benson (Scripps Institute of Oceanography) as well as severe pennate diatom contamination.

Juvenile Macrocystis used in our clean-room experiments were microscopically examined at the conclusion of each experiment. Only two out of the 300 experimental plants showed contamination. Both plants exhibited a small patch of black filamentous algae located at the blade tip.

#### 4.4 Seven Micronutrient Experiments, Series #20 - #90

##### 4.4.1 Introduction

The seven micronutrient experiments were each conducted with eight flasks and three plants per flask. Initial wet weights ranged from 4.5 to 5.5 mg, except for Series #20 when plants ranged from 6.5 to 7.5 mg. Each experiment lasted ten days. Paired flasks usually



contained identical Aquil formulations so that four concentrations of micronutrient were examined per series.

#### 4.4.2 Iron Experiment, Series #20

Four concentrations of iron as Fe(III) complexed with EDTA were examined. Two flasks each of 0, 10, 250 and 1000 nM Fe were tested. Plants in 250 nM Fe grew significantly faster than those in 0 nM Fe (Table 4.1). There were no significant statistical differences in growth rates between any of the other concentrations.

Table 4.2 shows elemental tissue data for individual plants in Series #20 for Fe, Mn, Cu and Zn, and shows overall ranges for As, Cr, Mo, Ni and Pb. Preliminary Experiment A, included for comparison (Table 4.3), shows values for eight flasks with identical media (500 nM Fe). Series #20 showed zinc levels of 0.83 to 1.21 ug-at per g dry wt. for plants grown in 0 nM Fe, while plants in 10 nM Fe showed only 0.43 to 0.58 ug-at per g dry wt. Zn. Tissue concentrations for iron ranged from 0.38 to 1.01 ug-at per g dry wt.

#### 4.4.3 Manganese Experiment, Series #30

Four manganese concentrations introduced as Mn(II) were studied in seven flasks. Concentrations were 0, 5, 25 and 100 nM Mn. Aquil SB had contained 10 nM Mn. Only one flask of 25 nM Mn was tested, while the eighth flask contained 0 nM Fe with 25 nM Mn.

Mean growth rates from the 5, 25 and 100 nM Mn cultures were all significantly higher than from 0 nM Mn (Table 4.4). Five nM Mn plants

Table 4.1 Growth rate data for iron experiment, series #20. Growth rates R (per day) of Macrocystis juveniles cultured in Aquil were converted to equivalent growth rates R' at 10°C using the temperature correction factor B.

[Fe] nM	Flask	Wet Weight, mg		Growth Rate, day <sup>-1</sup>			
		Day 1	Day 10	R	B	R'	
0	1	7	77	0.27	1.054	0.28	
		7	65	0.25		0.26	
		7	54	0.23		0.24	
	2	7	113	0.31	.989	0.30	
		7	85	0.28		0.27	
		7	79	0.27		0.27	
10	3	7	102	0.30	1.136	0.34	
		7	83	0.27		0.31	
		7	46	0.21		0.24	
	4	7	99	0.29	1.021	0.30	
		7	80	0.27		0.28	
		7	67	0.25		0.26	
	250	5	7	59	0.24	1.160	0.27
			7	63	0.24		0.28
			7	61	0.24		0.28
6		7	84	0.28	1.094	0.30	
		7	86	0.28		0.30	
		7	93	0.29		0.31	
1000	7	7	34	0.18	1.191	0.21	
		7	73	0.26		0.31	
		7	56	0.23		0.27	
	8	7	73	0.26	1.148	0.30	
		7	39	0.19		0.22	
		7	95	0.29		0.33	

Table 4.2 Iron experiment, series #20.  
 Elemental tissue concentrations  
 determined by XRF for Macrocystis  
 juveniles grown in Aquil SB with  
 Fe concentrations as indicated.

Plant No.	Aquil Concentration		Tissue Concentration, ug-at/g dry wt.			
	Fe, nM		Mn	Fe	Cu	Zn
F <sub>1</sub>	a	0	0.22	0.57	0.21	0.90
	b	0	<0.20	0.46	0.13	0.96
	c	0	<0.20	0.54	0.21	0.88
F <sub>2</sub>	a	0	<0.19	0.41	<0.09	0.86
	b	0	<0.21	0.56	0.20	0.83
	c	0	0.19	0.59	0.22	1.21
F <sub>3</sub>	a	10	0.28	0.43	<0.07	0.46
	b	10	<0.20	0.51	0.17	0.58
	c	10	<0.15	0.38	<0.07	0.46
F <sub>4</sub>	b	10	0.21	1.01	0.08	0.43
	c	10	<0.14	0.55	<0.06	0.56
Range			0.14- 0.28	0.41- 1.01	0.06- 0.22	0.43- 0.96

<u>Element</u>	<u>Range, ug-at/g dry wt.</u>
As	.05 - .10
Cr	<.20 - <.31
Mo	ND - -
Ni	<.08 - .26
Pb	<.04 - <.06
V	<.33 - <.52
Co	<.01 - --

Table 4.3 Preliminary experiment A. Elemental tissue concentrations determined by XRF for Macrocystis juveniles grown in Aquil with 25 nM Mn, 500 nM Fe, 5 nM Cu and 150 nM Zn.

Plant No.		Tissue Concentration, ug-at/g dry wt.			
		Mn	Fe	Cu	Zn
1	a	0.26	1.15	0.25	0.56
	b	0.24	0.86	0.20	0.42
	c	0.15	0.83	0.17	0.37
2	a	0.27	1.07	0.21	0.56
	b	0.20	0.82	0.17	0.44
	c	0.20	0.67	0.12	0.47
3	a	ND	0.65	0.19	0.23
	b	0.18	0.79	0.15	0.22
	c	0.26	0.64	0.21	0.32
4	a	ND	0.73	0.11	0.34
	b	ND	0.65	0.16	0.21
	c	ND	0.44	0.10	0.12
5	a	0.34	0.78	0.16	0.24
	b	0.26	0.70	0.14	0.22
	c	0.23	0.90	0.13	0.16
6	a	0.46	0.87	0.18	0.31
	b	0.16	0.78	0.11	0.31
	c	0.14	0.43	0.11	0.13
7	a	0.40	0.87	0.19	0.43
	b	0.19	1.07	0.16	0.46
	c	ND	0.61	0.12	0.38
8	a	0.21	0.47	0.18	0.20
	b	0.26	0.61	0.12	0.26
	c	0.12	0.49	0.13	0.23
nm conc in Aquil		25	500	5	150
Range		ND- 0.46	0.43- 1.15	0.10- 0.25	0.12- 0.56
<u>Element</u>	<u>Range, ug-at/g dry wt.</u>				
As	.05 - .10				
Cr	<.20 - <.30				
Mo	ND - -				
Ni	<.08 - .26				
Pb	<.04 - <.06				
V	<.33 - <.52				

Table 4.4 Growth rate data for manganese experiment, series #30. Growth rates R (per day) of *Macrocystis* juveniles cultured in Aquil were converted to equivalent growth rates R' at 10°C using the temperature correction factor B. Flask 1 contained 0 nM Fe, while other flasks contained 250 nM Fe.

[Mn] nM	Flask	Wet Weight, mg		Growth Rate, day <sup>-1</sup>		
		Day 1	Day 10 (Day 16)	R	B	R'
0	3	5.3	(73)	0.17	1.007	0.18
		5.5	(204)	0.24		0.24
		5.6	(298)	0.26		0.27
	4	4.5	(123)	0.22	1.058	0.23
		5.4	(154)	0.22		0.24
		5.6	(287)	0.26		0.28
5	5	4.5	70.2	0.30	1.038	0.32
		5.2	88.0	0.31		0.33
		5.4	105.1	0.33		0.34
	6	4.4	73.3	0.31	1.097	0.34
		5.2	76.0	0.30		0.33
		5.4	81.2	0.30		0.33
25	2	4.8	45.2	0.25	1.060	0.26
		5.0	83.2	0.31		0.33
		5.2	101.1	0.33		0.35
100	7	4.8	37.3	0.28	1.130	0.26
		5.1	50.2	0.25		0.27
		5.2	56.8	0.27		0.30
	8	4.7	53.0	0.27	1.071	0.29
		5.0	56.8	0.27		0.29
		5.1	67.3	0.29		0.31
25 (0 nM Fe)	1	4.1	(89)	0.20	1.002	0.20
		4.5	(93)	0.20		0.20
		4.6	(118)	0.22		0.22

also significantly outgrew the 100 nM plants. The 25 nM Mn (0 nM Fe) was the least successful Aquil formulation of the five tested in this series. Growth rates ranged from 0.17 per day (0 nM Mn) and 0.20 in 0 nM Fe, to 0.23 to 0.34 per day for the remaining media.

Tissue concentration of manganese (Table 4.5) did not increase as concentration of this element in Aquil was raised from 0 to 5, 25 and 100 nM Mn. Tissue Mn ranged between 0.12 and 0.42 ug-at per g dry wt. One plant contained 1.52 ug-at per g dry wt. Zn., while other plants had 0.19 to 0.56 ug-at per g dry wt.

#### 4.4.4 Copper Experiment, Series #40

Series #40 studied copper as Cu(II) at 0, 2.5, 10 and 50 nM with two flasks per concentration. Aquil SB contained 5 nM Cu. There were no statistical differences between mean growth rates from the different concentrations of copper (Table 4.6). Growth rates ranged from a poor 0.05 per day (for one plant in 0 nM Cu) up to 0.18 to 0.28 per day. One plant grown in a 0 nM Cu flask had a small amount of black filamentous alga on the blade tip. Tissue levels of copper remained between 0.04 and 0.20 ug-at per g dry wt. over an Aquil range of 0, 2.5, 10 and 50 nM Cu (Table 4.7).

#### 4.4.5 Zinc Experiment, Series #50

Aquil SB had contained 100 nM Zn, and one flask in series #50 received 1000 nM Zn in an attempt to induce a toxic reaction. The other levels were 0, 2, 10 and 200 (one flask) nM Zn. Plants in 1000 nM Zn

Table 4.5 Manganese Experiment, Series #30. Elemental tissue concentrations determined by XRF for *Macrocystis juveniles* grown in Aquil SB with Mn concentrations as indicated. Plants M<sub>1</sub> a, b and c were grown in 25 nM Mn.

Plant No.		Aquil	Tissue Concentration,			
		Concentration	ug-at/g dry wt.			
		Mn, nM	Mn	Fe	Cu	Zn
M <sub>3</sub>	a	0	.19	1.35	.22	.40
	b	0	.16	.67	.14	.32
	c	0	.12	.70	.18	.19
M <sub>4</sub>	a	0	.16	.83	.23	.30
	b	0	.18	.77	.29	.58
	c	0	.25	1.06	.20	.36
M <sub>5</sub>	a	5	.14	.91	.13	.42
	b	5	.25	.59	.18	.20
	c	5	.14	.91	.06	.19
M <sub>6</sub>	a	5	.14	.64	.24	.44
	b	5	.24	.91	.28	.53
	c	5	.25	1.39	.20	.28
M <sub>2</sub>	a	25	.27	1.10	.27	1.52
	b	25	.17	.74	.16	.48
	c	25	.34	.76	.15	.30
M <sub>7</sub>	a	100	.16	1.09	.15	.28
	b	100	.15	.91	.15	.28
	c	100	.42	.85	.17	.28
M <sub>8</sub>	a	100	.35	1.10	.19	.36
	c	100	.27	.96	.07	.32
Range			.12- .42	.59- 1.35	.07- .29	.19- 1.52

Element      Range, ug-at/g dry wt.

As	.02 - .27
Cr	.12 - .30
Mo	ND - -
Ni	.08 - .42
Pb	.02 - .12
V	.22 - .36

Plant No.		Aquil	Tissue Concentration,			
		Concentration	ug-at/g dry wt.			
		Fe, nM	Mn	Fe	Cu	Zn
M <sub>1</sub>	a	0	.18	1.12	.25	1.83
	b	0	.33	1.13	.09	1.63
	c	0	.21	.59	.16	1.32
Range			.18- .33	.59- 1.13	.09- .25	1.32- 1.83

Element      Range, ug-at/g dry wt.

As	.06 - .08
Cr	.18 - .27
Mo	ND - -
Ni	.09 - .16
Pb	.02 - .05

Table 4.6 Growth rate data for copper experiment, series #40. Growth rates R (per day) of *Macrocystis* juveniles cultured in Aquil were converted to equivalent growth rates R' at 10°C using the temperature correction factor B.

[Cu] nM	Flask	Wet Weight, mg		Growth Rate, day <sup>-1</sup>		
		Day 1	Day 10	R	B	R'
0	1	4.7	7.5	0.05	1.114	0.06
		4.9	26.8	0.19		0.21
		5.0	36.9	0.22		0.25
	2	5.1	38.7	0.22	1.128	0.25
		5.0	54.4	0.26		0.30
		5.3	55.9	0.26		0.29
2.5	3	5.0	40.4	0.23	1.128	0.26
		5.2	49.7	0.25		0.28
		5.4	50.1	0.25		0.28
	4	5.2	20.0	0.15	1.156	0.21
		5.4	24.0	0.17		0.19
		5.4	31.4	0.20		0.23
10	5	4.9	10.2	0.08	1.124	0.09
		5.0	28.5	0.19		0.22
		5.3	34.9	0.21		0.23
	6	4.7	32.3	0.21	1.108	0.24
		5.1	32.5	0.21		0.23
		5.3	33.0	0.20		0.22
50	7	4.6	15.3	0.13	1.102	0.15
		5.1	22.4	0.16		0.18
		5.2	23.6	0.17		0.18
	8	4.5	23.0	0.18	1.083	0.20
		4.5	37.3	0.23		0.25
		5.0	40.7	0.23		0.25



4.7 Copper experiment, series #40. Elemental tissue concentrations determined by XRF for Macrocystis juveniles grown in Aquil SB with Cu concentrations as indicated.

Plant No.		Aquil Concentration	Tissue Concentration, ug-at/g dry wt.				
		Cu,nM	Mn	Fe	Cu	Zn	Co
C <sub>2</sub>	a	0	.15	1.08	.11	.86	<.10
	b	0	.13	1.15	.11	1.19	<.10
C <sub>3</sub>	b	2.5	.20	2.12	.20	1.20	<.10
	c	2.5	.28	1.50	.05	1.17	
C <sub>5</sub>	c	10	.32	1.31	.14	1.31	<.10
C <sub>8</sub>	a	50	.16	.78	.05	.80	<.10
	b	50	.24	.99	.14	.79	<.10
	c	50	.14	.58	.04	.38	.10
Range			.13- .32	.78 2.12-	.04- .20	.38- 1.31	<.10- .10

Element	Range, ug-at/g dry wt.
As	.02 - .07
Cr	.12 - .20
Mo	ND - --
Ni	.03 - .27
Pb	.02 - .04
Co	<.01 - --

grew significantly faster during the 10 days than did plants in 2 nM Zn (Table 4.8). Growth rates ranged from a poor 0.09 per day (two plants in 0 nM Zn) to good rates of 0.20 to 0.26 per day for the other 22 plants. Plants from different levels of zinc exhibited varied morphology. The 1000 nM Zn plants were wrinkled while the 200 nM and 10 nM plants were very smooth, flat blades. The 2 nM plants were wrinkled at the base, while the 0 nM plants were very pale and smooth.

Plants showed increased tissue zinc concentration in the Aquil medium containing 1000 nM Zn (Table 4.9). The highest values obtained, 0.84 and 1.06 ug-at per g dry wt., occurred with 1000 nM Zn. The remaining plants ranged from 0.23 to 0.83 ug-at per g dry wt., which are considered normal.

#### 4.4.6 Cobalt Experiment, Series #60

Co was examined at three nanomolar levels: 0, 40, 300, and 300 (200 nM Cu). All formulations except 300 nM Co (200 nM Cu) in this series contained 5 nM Cu. Previously, a concentration of 100 nM Co had been used in Aquil SB.

Aquil amended with 300 nM Co (200 nM Cu) was a better medium than both 40 nM Co (5 nM Cu) and 300 nM Co (5 nM Cu) (Table 4.10). Growth rates were in the excellent performance range at 0.29 per day (0 nM Co) to 0.36 per day (300 nM Co). All plants appeared healthy.

The elemental tissue concentration for the cobalt experiment (Table 4.11) showed that plants grown in 300 nM Co plus 200 nM Cu did not take

Table 4.8 Growth rate data for zinc experiment, series #50. Growth rates R (per day) of *Macrocystis* juveniles cultured in Aquil were converted to equivalent growth rates R' at 10°C using the temperature correction factor B.

[Zn] nM	Flask	Wet Weight, mg		Growth Rate, day <sup>-1</sup>			
		Day 1	Day 10	R	B	R'	
0	1	4.9	11.4	0.09	.963	0.09	
		5.0	11.7	0.09		0.09	
		5.3	53.3	0.26		0.25	
	2	2	4.7	28.7	0.20	1.018	0.20
			4.9	31.7	0.21		0.21
			5.3	39.5	0.22		0.23
2	3	4.9	28.5	0.20	1.038	0.20	
		5.0	30.1	0.20		0.21	
		5.0	32.7	0.21		0.22	
	4	4	5.0	32.5	0.21	.993	0.21
			5.0	38.1	0.23		0.22
			5.3	42.9	0.23		0.23
10	5	4.7	14.3	0.12	.981	0.12	
		5.0	40.6	0.23		0.23	
		5.0	42.9	0.24		0.23	
	6	6	4.7	31.3	0.21	1.031	0.22
			5.1	34.4	0.21		0.22
			5.2	36.5	0.22		0.22
200	7	5.0	39.9	0.23	.988	0.23	
		5.2	40.9	0.23		0.23	
		5.4	43.7	0.23		0.23	
1000	8	4.6	36.0	0.23	1.023	0.23	
		4.7	38.2	0.23		0.24	
		4.8	46.1	0.25		0.26	

Table 4.9 Zinc experiment, series #50. Elemental tissue concentrations determined by XRF for Macrocystis juveniles grown in Aquil SB with Zn concentrations as indicated.

Plant No.	Aquil Concentration		Tissue Concentration, ug-at/g dry wt.			
	Zn, nM		Mn	Fe	Cu	Zn
Z <sub>1</sub>	a	0	.19	.73	.35	.50
Z <sub>2</sub>	a	0	.12	.62	.06	.23
	b	0	.09	.43	.10	.23
	c	0	.19	.91	.07	.42
Z <sub>4</sub>	b	2	.19	1.89	.12	.48
Z <sub>5</sub>	a	10	.27	.85	.15	.48
	b	10	.26	.75	.21	.35
Z <sub>6</sub>	a	10	.19	.96	.09	.83
	c	10	.13	.73	.19	.52
Z <sub>7</sub>	a	200	.17	1.15	.15	.51
Z <sub>8</sub>	b	1000	.12	.82	.10	.84
	c	1000	.17	.77	.08	1.06
Range			.09- .27	.43- 1.89	.06- .35	.23- 1.06

<u>Element</u>	<u>Range, ug-at/g dry wt.</u>
As	.03 - .08
Cr	.17 - .26
Mo	ND - --
Ni	.05 - .18
Pb	.03 - .06
V	.20 - .43
Co	<.01 - --

Table 4.10 Growth rate data for cobalt experiment, series #60. Growth rates R (per day) of *Macrocystis* juveniles cultured in Aquil containing 5 nM Cu. Flasks 7 and 8 were grown in 300 nM Co plus 200 nM Cu.

[Co] nM	Flask	Wet Weight, mg		Growth Rate, day <sup>-1</sup>
		Day 1	Day 10	R
0	1	4.5	63.7	0.29
		4.7	112.3	0.35
		4.6	78.1	0.31
	2	5.4	124.1	0.35
		4.6	82.7	0.32
		5.1	114.1	0.34
40	3	4.9	91.4	0.32
		4.5	83.4	0.32
		5.0	98.0	0.32
	4	4.6	92.4	0.33
		5.3	106.0	0.33
		5.1	98.2	0.33
300	5	4.9	74.6	0.30
		5.0	92.3	0.32
		5.2	133.1	0.36
	6	4.6	67.3	0.30
		4.8	68.5	0.29
		5.1	102.8	0.33
300 (200 nM Cu)	7	5.1	101.2	0.33
		5.3	110.9	0.34
		5.3	117.6	0.34
	8	5.0	114.3	0.35
		5.1	115.5	0.35
		5.2	118.0	0.35

Table 4.11 Cobalt series, experiment #60. Elemental tissue concentrations determined by XRF for Macrocystis juveniles grown in Aquil SB with Co concentrations as indicated and 5 nM Cu. Plants C<sub>7</sub> a,b,c and C<sub>8</sub> a,b, and c were grown in 200 nM Cu.

Plant No.	Aquil Concentration		Tissue Concentration, ug-at/g dry wt.				
	Co,nM	Mn	Fe	Cu	Zn	Co	
C <sub>1</sub> b	0	.16	1.24	.16	.42	<.010	
C <sub>2</sub> a	0	.20	.60	.12	.23	<.010	
C <sub>3</sub> c	40	.12	1.18	.11	.33	<.010	
C <sub>4</sub> c	40	ND	1.07	.12	.49	<.010	
C <sub>5</sub> b	300	.17	.77	.11	.38	<.010	
C <sub>6</sub> c	300	.11	1.04	.14	.39	<.010	
C <sub>7</sub>	a	300 (200 Cu)	.22	.95	.14	.39	<.010
	b	300 (200 Cu)	.10	.68	.17	.35	<.010
	c	300 (200 Cu)	.22	1.06	.12	.33	<.010
C <sub>8</sub>	a	300 (200 Cu)	.21	.79	ND	.26	<.010
	b	300 (200 Cu)	.30	.83	.11	.32	<.010
	c	300 (200 Cu)	.27	1.07	.15	.31	.015
Range		ND- .30	.60- 1.24	ND- .17	.23- .42	<.010- .015	

Element	Range, ug-at/g dry weight
As	.02 - .04
Cr	.15 - .24
Mo	.15 - .39
Ni	.05 - .14
Pb	.03 - .05
V	.16 - .40

up an increased amount of copper. Cobalt was  $<0.010$  ug-at per g dry wt. for 11 plants, and was  $0.015$  ug-at per g dry wt. in one plant grown in  $300$  nM Co ( $200$  nM Cu).

#### 4.4.7 Molybdenum Experiment, Series #70

Series #70 tested molybdenum at  $0$ ,  $100$ ,  $100$  with  $500$  nM Cu and  $1000$  nM. Mo was  $100$  nM in Aquil SB. The  $1000$  nM Mo plants developed significantly faster than either the  $0$  or  $100$  nM Mo (Table 4.12). Growth rates spanned from  $0.21$  to  $0.35$  per day for the two flasks containing  $100$  nM Mo.

The plant morphology from the molybdenum experiment was unusual and unique to the series. Blade tips from  $0$  nM Mo were very small and twisted  $360^\circ$  along the length of the blade. Three of the six  $100$  Mo plants formed narrow blades with edges rolled towards the center. The tips of three plants from  $100$  nM Mo ( $500$  nM Cu) were bent  $90^\circ$  to the major plane of the blade. All  $100$  nM Mo ( $500$  nM Cu) plants were smooth. The other three consisted of two plants with concave tips and one with a very short tip partially twisted around the axis of the length. The six plants in  $1000$  nM Mo were all large and smooth, four with concave tips, and two with very short twisted tips.

Only two plants from Series #70 were large enough for XRF analysis after plant pressing and dehydration (Table 4.13). Both of these plants were grown in  $500$  nM Cu. Molybdenum was detected at  $0.09$  and  $0.10$  ug-at per g dry wt., and copper was  $0.11$  ug-at per g dry wt. in both plants.

Table 4.12 Growth rate data for molybdenum experiment, series #70. Growth rates R (per day) of *Macrocystis* juveniles cultured in Aquil containing 5 nM Cu. Plants in flasks 5 and 6 were grown in 500 nM Cu.

[Mo] nM	Flask	Wet Weight, mg		Growth Rate, day <sup>-1</sup>
		Day 1	Day 10	R
0	1	5.2	73.0	0.29
		5.0	64.5	0.28
		4.7	35.0	0.22
	2	4.8	50.3	0.26
		4.9	60.4	0.28
		5.2	79.4	0.30
100	3	4.8	47.8	0.25
		4.8	69.4	0.30
		5.3	102.8	0.33
	4	4.9	47.6	0.25
		4.9	57.5	0.27
		5.0	71.0	0.29
100 (500 nM Cu)	5	5.2	35.1	0.21
		5.3	61.4	0.27
		5.5	78.6	0.29
	6	5.0	97.4	0.33
		5.0	106.0	0.34
		5.2	117.6	0.35
1000	7	5.0	79.6	0.31
		5.1	95.5	0.32
		5.2	102.0	0.33
	8	4.8	78.4	0.31
		5.1	85.2	0.31
		5.3	98.1	0.32



Table 4.13 Molybdenum series, experiment #70. Elemental tissue concentrations determined by XRF for Macrocystis juveniles grown in Aquil SB with Mo concentrations as indicated, and 500 nM Cu.

<u>Plant No.</u>		<u>Aquil</u>	<u>Mn</u>	<u>Tissue Concentration,</u>			
		<u>Concentration</u>		<u>ug-at/g dry wt.</u>			
		<u>Mo, nM</u>		<u>Fe</u>	<u>Cu</u>	<u>Zn</u>	<u>Mo</u>
M <sub>6</sub>	a	100 (500 nM Cu)	.19	.66	.11	.43	.09
M <sub>6</sub>	c	100 (500 nM Cu)	.39	.52	.11	.49	.10
Range			.19-	.52-	--	.43-	.09
			.39	.66	.11	.49	.10

<u>Element</u>	<u>Range, ug-at/g dry wt.</u>
As	.03 --
Cr	.17 - .18
Ni	.05 - .10
Pb	.04 --
V	.29 - .30
Co	<.01 - --

#### 4.4.8 Iodine Experiment, Series #80

Series #80 differed from our other micronutrients series by using two chemical forms of iodine, the element under examination, iodide ( $I^-$ ) and iodate ( $IO_3^-$ ). Besides 0 nM I, two iodide concentrations, 100 and 500 nM  $I^-$  and one iodate concentration, 100 nM  $IO_3^-$  were tested. There were no significant differences between mean growth rates of the various iodine concentrations (Table 4.14). Growth rates ranged from low values of 0.21 per day (500 nM  $I^-$ ) and 0.24 (0 nM I) to a high value of 0.30 per day in 100 nM  $I^-$ . Aquil SB had been enriched with a concentration of 100 nM  $I^-$ .

Plant morphology provided revealing information regarding effects from the series. Plants from 0 nM I were extremely brittle with tears, shot holes and paling tips. These were indications of rapidly failing health. Small pieces of sloughed tissue were also present in the media. Wrinkled tissue with curls, twists, tears and holes characterized the 100  $IO_3^-$  plants. Unhealthy morphological signs did not appear among plants in 100 nM  $I^-$ . Curled tissue was a predominant characteristic among plants grown in 500 nM  $I^-$  media.

Routine XRF analysis at Crocker Nuclear Laboratory did not include iodine, but special iodine analyses were performed for Series #80 (Table 4.15). Tissue concentrations ranged between 0.86 and 1.28 ug-at per g dry wt. or 0.011 to 0.016% I by weight.

Table 4.14 Growth rate data for iodine experiment, series #80.  
Growth rates R (per day) of Macrocystis juveniles  
cultured in Aquil.

[I] nM	Flask	Wet Weight, mg		Growth Rate
		Day 1	Day 10	day <sup>-1</sup> R
0 I <sup>-</sup>	1	4.7	42.6	0.24
		4.9	51.2	0.22
		5.0	80.9	0.30
	2	4.8	47.6	0.25
		4.8	74.3	0.30
		5.0	95.3	0.32
100 IO <sub>3</sub> <sup>-</sup>	3	4.7	61.1	0.28
		4.8	71.0	0.29
		5.4	80.9	0.29
	4	4.7	58.7	0.27
		5.0	87.1	0.31
		5.3	87.3	0.30
100 I <sup>-</sup>	5	5.0	33.2	0.21
		5.0	73.1	0.29
		5.2	85.9	0.30
	6	4.7	45.9	0.25
		5.0	61.0	0.27
		5.4	61.6	0.26
500 I <sup>-</sup>	7	4.5	36.4	0.23
		5.2	37.1	0.21
		5.4	72.8	0.28
	8	4.6	52.6	0.26
		4.8	58.3	0.27
		4.9	80.7	0.30

Table 4.15 Iodine experiment, series #80. Elemental tissue concentrations determined by XRF for Macrocystis juveniles grown in Aquil SB with I concentrations as indicated.

Plant No.	Aquil Concentration		Tissue Concentration, ug-at/g dry wt.				
	I, nM		Mn	Fe	Cu	Zn	I
I <sub>1</sub> a	0		ND	1.83	.15	.21	.86
I <sub>3</sub> b	100 10 <sup>-3</sup>		.39	3.22	.27	.40	1.28
I <sub>4</sub> b	100 10 <sup>-3</sup>		.25	1.31	.19	.21	1.08
c	100 10 <sup>-3</sup>		ND	1.82	.19	.75	.95
I <sub>5</sub> a	100 I <sup>-</sup>		.32	1.10	.18	.15	1.10
I <sub>7</sub> c	500 I <sup>-</sup>		ND	1.54	.17	.20	1.26
I <sub>8</sub> a	500 I <sup>-</sup>		ND	2.20	.16	.30	1.06
Range			ND- .39	1.10- 3.22	.15- .27	.15- .75	.86- 1.28

Element	Range, ug-at/g dry wt.
As	.02 - .06
Cr	.05 - .28
Mo	.17 - .75
Ni	.07 - .29
Pb	.04 - .06
V	.15 - .46
Co	<.01 - --

#### 4.4.9 Micronutrient Deficiency Experiment, Series #90

Seven differing flasks of Aquil SB were prepared. Each of the seven formulations lacked one of the micronutrients tested in Series #20 through #80; Co, Cu, I, Fe, Mn, Mo, and Zn. Media were changed every three days for 33 days, and there were three plants per flask.

Growth rates and increase over initial weight, ( $W_f/W_i$ ) are shown in Table 4.16. There were no differences between growth rates, which averaged a poor 0.10 per day. By day 25 of Series #90, one Mn and one Fe plant had small paling spots. In addition to paling spots, Co, Mo and Zn kelp had either a few small pieces or very fine particles of tissue floating in the media. By day 28 all plants had pale areas and were sloughing tissue. I, Mo and Zn juveniles had degenerated further and showed clear areas. On day 31, juveniles appeared limp, and clear spots and pale areas continued to spread. After 33 days, plants still had healthy-colored dark brown basal tissue, but the rest of the blade tissue had degenerated. Although there were some dark areas at the tips, in general the blades were characterized by shot holes, pale sections and clear spots where pigmented tissue had sloughed completely.

It was not possible to determine the exact day of death of these plants, as health very gradually declined and was without sudden dissolving of the tissue as was seen in preliminary experiments. Based on morphological signs of health, the plants can be ranked in order of decreasing health according to the deficient micronutrient: Fe > Cu > Mn > Co > I > Mo > Zn.

Table 4.16 Growth rate data for micronutrients deficiency experiment, series #90. Growth rates R (per day) of Macrocystis juveniles cultured in Aquil, and increase in weight in 33 days,  $W_f/W_i$ .

[M], 0 nM	Flask	Wet Weight, mg		Growth Rate,	Increase in
		Day 1	Day 33	Day $\frac{-1}{-1}$ R	Weight $\frac{W_f}{W_i}$
Fe	1	4.5	80.0	0.09	17.8
		4.5	123.5	0.10	27.4
		4.5	142.3	0.11	31.6
Cu	2	4.5	140.0	0.11	31.1
		4.9	152.0	0.11	31.1
		5.2	162.6	0.11	31.3
Mn	3	4.5	111.2	0.10	24.7
		4.5	140.4	0.11	31.2
		4.8	151.8	0.11	31.6
Zn	4	5.0	93.2	0.09	18.6
		5.0	111.2	0.10	22.2
		5.0	116.4	0.10	23.3
Mo	5	4.5	104.0	0.10	23.1
		4.7	104.8	0.10	22.3
		4.7	115.8	0.10	24.6
I	6	4.9	112.4	0.10	22.9
		5.1	139.6	0.10	27.4
		5.1	140.0	0.10	27.4
Co	7	4.8	96.2	0.09	20.0
		5.0	119.0	0.10	23.8
		5.0	137.5	0.10	27.5

XRF analysis of Series #90 (Table 4.17) showed average levels of Fe, Mn, Cu and Zn. Plants grown in 0 nM Fe had elevated levels of Zn, 1.19 and 2.47 ug-at per g dry wt., as seen previously in Series #20.

Table 4.17 Micronutrient deficiency experiment, series #90.  
Elemental tissue concentrations determined by XRF  
for Macrocystis juveniles grown in Aquil SB with  
each flask deficient in one of seven micronutrients.

Plant No.	Deficient Micronutrient	Tissue Concentration, ug-at/g dry wt.			
		Fe	Mn	Cu	Zn
Fe <sub>1</sub>	a	.88	.25	.21	1.19
	b	.78	.33	.32	2.47
Mn <sub>3</sub>	a	1.54	.41	.21	.32
	b	.89	.24	.19	.28
	c	.66	.19	.16	.24
Cu <sub>2</sub>	a	.71	.20	.21	.26
	b	.69	.20	.19	.25
	c	.87	.40	.07	.47
Zn <sub>4</sub>	a	1.8	.25	.15	.24
	b	2.52	.37	.18	.22
	c	1.85	.42	.54	.61
Co <sub>8</sub>	a	1.36	.20	.07	.32
	b	1.43	.25	.08	.31
	c	1.65	.25	.18	.43
Mo <sub>5</sub>	a	1.48	.31	.28	.26
	b	1.39	.24	.20	.19
	c	1.42	.28	.22	.33
I <sub>6</sub>	a	.76	.21	.26	.29
	b	1.36	.23	.26	.40
	c	1.58	.19	.20	.30

Element	Range, ug-at/g dry wt.
As	.02 - .08
Cr	.14 - .52
Mo	.14 - .41
Ni	.09 - .30
Pb	.05 - .12
V	.22 - .87
Co	-- - --



CHAPTER 5  
DISCUSSION OF RESULTS

5.1 Iron Reduction by Macrocystis Juveniles

5.1.1 Rate of Iron Reduction per Surface Area

Large-sized juvenile sporophytes of Macrocystis pyrifera reduced  $9.14 \times 10^{-11}$  moles of Fe(III) per  $\text{cm}^2$  per day on the last day of the experiment, when changes in tissue area were assumed to be small. Owens and Chaney (1971) estimated that the fresh water green alga Chlorella sorokiniana reduced 14 umoles of Fe(III) per  $10^8$  cells per hour. For an average cell diameter of 5  $\mu\text{m}$  (Brock, 1974) this is equivalent to  $2.14 \times 10^{-11}$  moles Fe(III) per  $\text{cm}^2$  per day. Thus juvenile kelp reduced over four times the amount of iron processed by the green alga.

5.1.2 Rate of Reduction Relative to Uptake Requirements

Based on Equation 2.1, biomass increased 530 mg on the last day of the experiment. The amount of iron incorporated into new biomass was 3.2 nmoles of Fe, based on tissue iron concentration determined by XRF. Juveniles reduced 40.3 nmoles Fe(III), excluding photoreduction, during the last 24 hours. This was approximately 12 times more than needed for the new tissue.

Because reduction occurs at lower rates in darkness, iron reduction may not be governed by plant nutritional requirements. It may be a

passive process that continues independently.

## 5.2 Micronutrients Experiments

### 5.2.1 Comparison of Growth Rate Data

Growth rates obtained in the cleanroom experiments using the synthetic seawater Aquil were much larger than those reported by North and Wheeler (1977) using enriched natural seawater (Table 5.1). The difference in growth rates was primarily due to the much smaller sizes and younger ages of plants used in the Aquil studies. (As juveniles mature, growth rates decrease, as discussed in Chapter 2). Illumination and availabilities of micronutrients were secondary factors. Light was at a saturating level in the cleanroom experiments and growth rates up to 0.38 per day were obtained using Aquil formulations. Differences in measuring techniques prevent comparing growth rates between Macrocystis gametophytes (Kuwabara, 1980) and juvenile sporophytes.

### 5.2.2 Micronutrient Concentrations Producing Optimal Growth

Only copper (Series #40) and iodine experiments (Series #80) failed to produce either stimulatory or inhibitory responses from juvenile kelp. This indicates that toxic levels had not been reached at the highest concentrations used and that reserves were not depleted after 10 days of growth in the absence of copper or iodine. (Table 5.2, after Kuwabara (1980)). Kuwabara (1980) used a multivariate approach and found the optima in Aquil for Macrocystis gametophytes at 5 nM Cu and 100 nM I<sup>-</sup> (Table 5.3). Macrocystis juvenile sporophytes appear

Table 5.1 Comparison of growth rates between juvenile Macrocystis.  
 R1 for North and Wheeler (1977) enriched natural seawater.  
 R2 Aquil formulations.

<u>Growth Rate, per day</u>		<u>Plant state of health</u>
<u>R1</u>	<u>R2</u>	
<u>Enriched seawater</u>	<u>Aquil formulations</u>	
>0.15	>0.35	excellent
0.10 - 0.15	0.25 - 0.35	good
0.05 - 0.10	0.05 - 0.25	satisfactory (fair)
<0.05	0.05 - 0.15	poor

Table 5.2 Growth rate results of the seven micronutrients experiments, Series #20-90 for juvenile Macrocystis grown in the artificial seawater Aquil with background micronutrient concentrations of Aquil SB. Results are indicated at which micronutrient levels juveniles grew significantly faster (>) and at levels neither stimulatory nor inhibitive (=). When no designation is given, levels were neither stimulatory nor inhibitive.

Series No.	Micro-nutrient	Concentrations Tested, nM	Aquil SB, nM	Results
20	Fe	0, 10, 250, 1000	250	250>0
30	Mn	0, 5, 25, 100	10	5, 25, 100>0, 5>100
40	Cu	0, 2.5, 10, 50	5	0=2.5=10=50
50	Zn	0, 2, 10, 200, 1000	100	1000>2
60	Co	0, 40, 300, 300 (200 Cu)	100	300 (200 Cu)>40, 300 (200 Cu)>300
70	Mo	0, 100, 1000, 100 (500 Cu)	100	1000>0, 1000>100
80	I	0, 100, 500, 100 (IO <sub>3</sub> <sup>-</sup> )	100	0=100 I <sup>-</sup> =100 IO <sub>3</sub> <sup>-</sup> = 500 I <sup>-</sup>
90	0	0Fe, 0Mn, 0Cu, 0Zn, 0I, 0Mo, 0Co		0Fe>0Cu>0Mn>0Co> 0I>0Mo>0Zn (morphologically determined)

Table 5.3 Comparison of trace element constituents ( $\mu\text{M}$ ) among a variety of marine growth media (after Kuwabara). Trace element data for synthetic media developed by other research were from Stein (1973). Formulations A through D represent work by Provasoli (1963) and Iwasaki (1967), Droop in Provasoli *et al.* (1957), Müller (1962), and McLachlan (unpublished), respectively. Values for seawater were from Brewer (1975). Macrocystis gametophyte Aquil formulation by Kuwabara (1980).

Element	<u>Synthetic Media</u>				<u>Aquil Formulations</u>		
	A	B	C	D	Sea- water	<u>Macrocystis</u> Gametophyte Multivariate Optimum	<u>Macrocystis</u> Juvenile Monovariate Optima
Br	125	275	185	500	840	800	800
Sr	23	44	14	100	91	63.8	63.8
Al	-	1	0.21	-	0.07	-	-
Rb	2.3	0.7	0.13	2	1.4	-	-
Li	28.8	0.87	0.14	10	26	-	-
I	0.08	0.16	0.12	0.2	0.5	0.10	0.10
Zn	0.8	35.2	14	35	0.076	0.17	1.00
Mn	7.3	11.8	3.8	10	0.0036	0.03	0.005
Mo	-	2.1	0.83	5	0.1	0.10	1.00
Co	0.17	0.11	0.04	0.3	0.0008	0.07	0.30
Cu	-	0.02	0.008	0.3	0.008	0.01	0.005
Fe	1.8	1.8	1.2	2	0.035	0.35	0.250
B	185	-	32	400	410	490	490
Na <sub>2</sub> EDTA	26.9	53.7	53.7	48	-	6.0	5.13

to be less sensitive than the microscopic gametophytic stages to copper and iodine.

The highest levels tested of zinc, cobalt and molybdenum produced the fastest growth rates. Kuwabara (1980) found optima for Macrocystis gametophytes at 170 nM Zn, 100 nM Mo and 70 nM Co (Table 5.3). It was found that juvenile kelp grew fastest at higher concentrations for all three elements; 1000 nM Zn, 1000 nM Mo and 300 nM Co.

Iron Experiments, Series #20, showed Aquil SB with 250 nM Fe was an improved formulation over Aquil SB with zero iron. Five, 25 and 100 nM Mn all proved to be superior to 0 nM Mn in the manganese experiment, Series #30. Manganese experiments gave faster growth rates for 5 nM Mn compared to 100 nM Mn.

Kelp grew faster in manganese at 5, 25 and 100 nM than at 0 nM Mn. 250 nM Fe also proved a better formulation than 0 nM Fe, as did 1000 nM Mo compared to 0 nM Mo. Juvenile Macrocystis is apparently quite sensitive to iron, manganese and cobalt deprivation, and also to higher levels of manganese.

Juvenile morphology (see Chapter 2, Figure 2.10) was used to rank the plants as to healthiness in the Micronutrient Deficiency Experiment, Series #90. The decreasing ranking of health according to the deficient micronutrient was: Fe > Cu > Mn > Co > I > Mo > Zn. The actively growing tissue at the base of the blade remained a dark, healthy brown as the health of the remained of each blade gradually declined.

Possibly the stressed blade transported remaining stores of nutrients and energy from the rest of the blade to the basal region to preserve this critical tissue.

These results imply that juvenile Macrocystis store quantities of micronutrients, sufficient for 20- to 30-fold increases in weight (see Section 5.3.12). Such unanticipated large reserves obviously interfered with attempts to ascertain the optimal levels and establish requirements for micronutrients in the ten-day experiments.

Growth rate data for juvenile Macrocystis cultured in enriched natural seawater were not compared with values from Aquil culturing. Results from seawater usually failed to show whether addition of metals increased growth rates, because light intensity was not at a saturating level (North, personal communication). North found growth inhibition in seawater cultures from 100 nM copper and occasionally from 1000 nM zinc.

### 5.2.3 Free Ion Concentrations in Aquil

The computer program REDEQL2 was used to calculate the free micronutrient ion concentrations in the various Aquil formulations (Table 5.3a). Total iron was varied over three orders of magnitude (0 to 1000 nM), while the free ion concentration ( $\text{Fe}^{3+}$ ) varied from  $5.7 \times 10^{-20}$  M to  $7.9 \times 10^{-20}$  M. The EDTA that was added to keep iron in a dissolved state also prevented the free iron levels from changing. The free ion levels changed at least one order of magnitude for Mn, Cu, Zn, Co, Mo and I. Mo and I existed completely in the ionic forms,

Table 5.3a Free micronutrient ion concentrations of various Aquil formulations as calculated by REDEQL2.

Element	$-\log [Element]$	$[Element]$ , nM	$[Fe^{3+}]$	$[Mn^{2+}]$	$[Cu^{2+}]$	$[Zn^{2+}]$	$[Co^{2+}]$	$[I^-]$	$[MoO_4^-]$
Fe	8.0	10	19.10	8.52	12.10	8.55	8.70	7.0	7.0
	6.6	250	19.23	8.90	13.68	10.18	10.28	7.0	7.0
	6.0	1000	19.24	9.35	14.68	10.78	10.88	7.0	7.0
Mn	8.3	5	19.23	9.20	13.68	10.17	10.28	7.0	7.0
	8.0	10	19.24	8.90	13.68	10.17	10.28	7.0	7.0
	7.6	25	19.23	8.50	13.68	10.17	10.27	7.0	7.0
	7.0	100	19.22	7.89	13.68	10.17	10.27	7.0	7.0
Cu	8.6	2.5	19.23	8.91	13.98	10.18	10.28	7.0	7.0
	8.3	5	19.23	8.91	13.68	10.17	10.28	7.0	7.0
	8.0	10	19.23	8.91	13.38	10.18	10.28	7.0	7.0
	7.3	50	19.23	8.91	12.68	10.17	10.28	7.0	7.0
Zn	8.7	2	19.24	8.91	13.69	11.89	10.29	7.0	7.0
	8.0	10	19.24	8.91	13.69	11.19	10.29	7.0	7.0
	6.7	200	19.22	8.90	13.67	9.87	10.27	7.0	7.0
	6.0	1000	19.14	8.85	13.59	9.09	10.27	7.0	7.0
Co	7.4	40	19.23	8.90	13.68	10.18	10.28	7.0	7.0
	6.52	300	19.21	8.89	13.66	10.15	9.38	7.0	7.0
	6.52	300	19.19	8.88	12.04	10.14	9.35	7.0	7.0
	(Cu,6.7,	200							
Mo	7.0	100	19.23	8.90	13.68	10.18	10.28	7.0	7.0
	6.0	1000	19.23	8.90	13.68	10.18	10.28	7.0	6.0
	7.0	100	19.18	8.87	11.63	10.13	10.23	7.0	7.0
(Cu,6.3,	500)								
I	7.0	100	19.23	8.90	13.68	10.18	10.28	7.0	7.0
	6.3	500	19.23	8.90	13.68	10.18	10.28	6.3	7.0
Aquil SB, [EDTA]=5.3			19.23	8.90	13.68	10.17	10.28	7.0	7.0
Total Element Concentration			6.6 <sub>T</sub>	8.0 <sub>T</sub>	8.3 <sub>T</sub>	7.0 <sub>T</sub>	7.4 <sub>T</sub>	7.0 <sub>T</sub>	7.0 <sub>T</sub>



$\text{MoO}_4^-$  and  $\text{I}^-$ .

### 5.3 Analysis of XRF Data

Tissue metal concentration results for four trace metals from Series #20 through #80 were examined for unusually high concentrations with respect to means and ranges (Table 5.4), to identify possible contamination and plant-media interactions. Average elemental concentrations for 21 elements are shown in Table 5.5.

#### 5.3.1 Interactive and Non-Interactive Zones

Tissue metal concentration data for juveniles grown in enriched natural seawater (North 1980) (Table 5.6) provided the basis against which tissue concentrations from Aquil cultures could be compared. Reactions of the kelp to Cu, Mn, Fe and Zn additions to either seawater or to Aquil fell into two categories; interactive and non-interactive. Increases in media micronutrient concentrations were accompanied by corresponding increases of micronutrients in the tissues for interactive categories. Non-interactive categories include those plants that maintained constant tissue concentrations regardless of micronutrient concentrations in the medium.

Both categories occurred in North's experiments. Manganese in tissues was constant (non-interactive) regardless of seawater additions from 0 to 1000 nM Mn. Plant composition remained unchanged for iron additions up to and including 1000 nM (non-interactive). Tissue concentrations increased at 10,000 nM Fe (interactive). Plants were

Table 5.4 Comparison of concentrations of Fe, Mn, Cu and Zn in ug-at/g dry weight, determined by XRF, found among juvenile Macrocystis plants grown in a kelp farm (North, pers. comm.) and those cultured in the artificial seawater Aquil.

Tissue Concentration Category	Tissue Concentration, ug-at/g dry weight			
	<u>Fe</u>	<u>Mn</u>	<u>Cu</u>	<u>Zn</u>
<u>Aquil-grown Juveniles</u>				
Average	1.05	0.21	0.15	0.37
Normal range	0.41-1.54	ND-0.46	ND-0.35	0.21-0.58
Above normal	>2.00	--	--	>0.83
Below normal	--	--	--	<0.19
<u>Kelp Farm Juveniles</u>				
Range	1.83-11.91	ND	ND-1.08	0.45-2.92

Table 5.5 Average elemental concentrations of juvenile Macrocystis grown in artificial seawater, Aquil, determined by XRF.

<u>Element</u>	<u>Tissue Concentration ug-at/g dry weight</u>
I	1.86
Co	<0.10
Mn	0.21
Fe	1.05
Cu	0.15
Zn	0.37
Mo	0.42
S	47.81
Cl	1415.12
K	1371.28
Ca	226.53
Ti	0.59
V	0.31
Cr	0.19
Ni	0.12
As	0.04
Pb	0.04
Br	2.29
Rb	0.05
Sr	4.24
Zr	0.07

Table 5.6 Effects on tissue concentrations in individual large juvenile *Macrocystis* sporophytes from culturing for one week in seawater enriched as indicated by North (1980).

Element tested							
Cu		Zn		Fe		Mn	
$\mu\text{M}$ in medium	$\frac{\mu\text{g-at}}{\text{g dry wt}}$	$\mu\text{M}$ in medium	$\frac{\mu\text{g-at}}{\text{g dry wt}}$	$\mu\text{M}$ in medium	$\frac{\mu\text{g-at}}{\text{g dry wt}}$	$\mu\text{M}$ in medium	$\frac{\mu\text{g-at}}{\text{g dry wt}}$
Control <sup>a</sup>	ND <sup>b</sup> 0.06 0.09	Control	1.50 1.78 2.00 1.76	Control	1.76 1.90 1.76 1.28	Control	0.28 0.58 0.28 0.34
Mean	0.075		1.76		1.68		0.37
(SD)	(0.02)		(0.20)		(0.27)		(0.14)
0.01	0.08 ND ND	0.1	4.65 4.13 3.63 4.36	0.1	1.48 1.68 1.56 1.97	0.01	0.26 0.45 0.35 0.43
Mean	0.08		4.19		1.67		0.37
(SD)			(0.43)		(0.21)		(0.09)
0.05	0.04 0.12 0.25	1.0	17.97 19.56 17.10 7.87	1.0	1.82 1.41 1.48 1.60	0.10	0.39 0.43 0.39 0.26
Mean	0.14		15.63		1.58		0.37
(SD)	(0.11)		(5.26)		(0.18)		(0.07)
0.1 (surface)	0.15 0.14 0.12	10.0	7.15 14.74 15.51 9.01	10.0	5.18 3.88 4.53 3.37	1.00	0.25 0.15 0.22
Mean	0.14		11.60		4.24		0.21
(SD)	(0.02)		(4.14)		(0.79)		(0.05)
0.2	0.41 0.34 0.19 0.19						
Mean	0.28						
(SD)	(0.11)						
0.4 (surface)	0.73 1.31 0.93						
Mean	0.99						
(SD)	(0.30)						

<sup>a</sup> Seawater media all collected from 300-m depths unless otherwise indicated.

<sup>b</sup> ND = not detected.

always in the interactive category when seawater was supplemented with zinc as high as 10,000 nM. Consequently, tissue concentrations well above background values might reflect zinc contamination in the system. Copper concentrations were in the interactive zone from 50 to 400 nM Cu. Juveniles collected from a kelp farm 6 km offshore from Corona del Mar showed a zinc range from 0.45 to 2.92 ug-at per g dry wt. (Table 5.4).

#### 5.3.2 Categories for Tissue Metal Concentration (Table 5.4)

Tissue metal concentrations for Aquil-grown plants can be divided into normal, above-normal and below-normal categories. Juveniles collected from a kelp farm 6 km offshore from Corona del Mar provided a comparison. Average concentration in Aquil-grown plants in ug-at per g dry wt. were: 1.05 Fe, 0.21 Mn, 0.15 Cu and 0.37 Zn. The above-normal category began at 2.00 ug-at per g dry wt. Fe and 0.83 ug-at per g dry wt. Zn. Values below 0.19 ug-at per g dry wt. Zn comprised the below-normal category. Kelp farm juveniles had larger ranges of Fe and Zn tissue concentrations than Aquil-grown kelp.

#### 5.3.3 Iron Experiment, Series #20, and Preliminary Experiment A

All six plants grown in 0 nM Fe in the Iron Experiment, Series #20, showed above normal zinc levels, from 0.83 to 1.21 ug-at per g dry wt. (see Table 4.2). Normal tissue zinc ranged between 0.21 and 0.58 ug-at per g dry wt. (Table 5.4). Plants grown in 10 nM Fe showed normal levels of zinc. Either iron deficiency stimulated increased zinc uptake or a minor degree of contamination occurred. Levels of Fe, Mn and Cu were all within the normal ranges. Preliminary Experiment A (500 nM

Fe) showed normal values of Fe, Cu and Zn (see Table 4.3). These results identify the iron concentration range of 0 to 500 nM as belonging to the non-interactive category. Data by North (1980) showed a non-interactive zone below 10,000 nM (Table 5.6). However, plants grown in seawater enriched with iron below 10,000 nM had tissue iron concentrations averaging higher than Aquil-grown plants at 1.68 ug-at per g dry wt. for seawater vs. 0.37 ug-at per g dry wt. for Aquil.

#### 5.3.4 Manganese Experiment, Series #30

Tissue metal concentration data showed that the Manganese Series, Experiment #30, was in the non-interactive category (see Table 4.5). Tissue concentrations of Fe, Cu and Zn were within the ranges of normal tissues except that one juvenile out of the 20 contained zinc at 1.52 ug-at per g dry wt. Levels of tissue manganese were similar for enriched seawater (North, 1980) (Table 5.6), and Aquil.

Three plants exposed to 0 nM Mn showed above normal levels of zinc, as in Series #20. Iron tissue levels ranged from 1.32 to 1.83 ug-at per g dry wt., compared to the normal range of 0.30 to 0.70 ug-at per g dry wt.

#### 5.3.5 Copper Experiment, Series #40

All kelp plants grown in 0, 2.5, 10 and 50 nM Cu (Series #40) showed normal tissue copper levels, and thus were classed as non-interactive (see Table 4.7). Zinc values exceeded the normal tissue range, indicating probable zinc contamination. Plants may have retained iron while being prepared for XRF analysis or during the analysis

procedure. One plant out of the eight analyzed by XRF showed a high iron concentration of 2.12 ug-at per g dry wt. Otherwise, copper and iron concentrations were normal. Levels of tissue copper for comparable copper concentrations in enriched seawater (North, 1980) (Table 5.6) and Aquil were similar to the values from Series #40. Results for higher copper concentrations in Aquil are discussed below (Series #60 and #70).

#### 5.3.6 Zinc Experiment, Series #50

Kelp plants grown in 0, 2, 10, 200 and 1000 nM Zn in Series #50 were analyzed by XRF. Tissue zinc values for 0, 2, 10 and 200 nM Zn were in the normal range, but the two 1000 nM Zn plants large enough for analysis contained above normal zinc at 0.84 and 1.06 ug-at per g dry wt. (see Table 4.9). An interactive status at 1000 nM Zn agrees with North's (1980) data, but his values for 1000 nM Zn were 15 times greater than the two Aquil plants. Comparison between tissue zinc concentrations in juveniles collected from a kelp farm (Table 5.4) as well as juveniles cultured in seawater (Table 5.6) both showed higher zinc ranges than for Aquil: 0.45 to 2.92 ug-at per g dry wt. Zn for ocean plants, vs. 1.50 to 2.00 ug-at per g dry wt. Zn for seawater-cultured plants from Corona del Mar, and 0.21 to 0.58 ug-at per g dry wt. Zn for Aquil-grown plants. All kelp tissue concentrations for Fe, Mn and Cu in Series #50 were within the normal range.

#### 5.3.7 Cobalt Experiment, Series #60

The Cobalt Experiment was free from Fe, Cu and Zn contamination as indicated by normal tissue concentration ranges (see

Table 4.11). Six plants grown in 300 nM Co plus 200 nM Cu did not show elevated tissue copper. Plants were previously shown in Series #40 to be in the non-interactive range for 0 to 50 nM Cu. Series #60 results extend the non-interactive category up to 200 nM Cu. North's data (1980) indicated that the interactive category for copper in seawater (with a background of 3 to 10 nM Cu) began at supplementations of 100 nM Cu. (Table 5.6).

#### 5.3.8 Molybdenum Experiment, Series #70

Two plants from the Molybdenum Experiment were sufficiently large after culturing to be analyzed by XRF (Table 4.13). Both were grown in 100 nM Mo with 500 nM Cu. Tissue molybdenum concentrations were 0.09 and 0.10 ug-at per g dry wt. Cu, Fe and Zn were all within normal ranges. The non-interactive category for copper was again extended, up to 500 nM Cu. Tissue molybdenum concentrations were 0.09 and 0.10 ug-at per g dry wt.

#### 5.3.9 Iodine Experiment, Series #80

There were no significant differences in tissue levels of iodine for plants grown in 0 nM I, 100 nM  $\text{IO}_3^-$ , 100 nM  $\text{I}^-$  or 500 nM  $\text{I}^-$  (see Table 4.15). Tissue levels of iron exceeded the normal range. Aquil SB media were designed to contain 250 nM Fe, but tissue levels indicated a concentration above 500 nM, using Experiment A results, and well above 1000 nM Fe using North's (1980) data. This seems unlikely, so the cause of the high tissue-Fe values remains mysterious. Series #80 was clean with regard to Mn, Cu and Zn



contamination.

#### 5.3.10 Micronutrient Deficiency Experiment, Series #90

Elemental tissue concentrations were determined by XRF for plants grown in seven formulations of Aquil, each deficient in one of the seven micronutrients: Fe, Mn, Cu, Zn, Co, Mo and I (Table 4.17). Levels of metals were comparable with other test plants grown in complete formulations of Aquil (Table 5.5).

Plants grown in Aquil with no added iron showed elevated zinc levels (1.19 and 2.47 ug-at per g dry wt. when compared to other plants in Series #90 (an average 0.32 ug-at per g dry wt.) and the average level in other Aquil-cultured plants (0.37 ug-at per g dry wt.). This is consistent with the findings from the 0 nM Fe plants in Series #20.

#### 5.3.11 Comparison of Aquil-Cultured and Ocean-Collected Juveniles

Juveniles collected from a kelp farm 6 km offshore of Corona del Mar showed higher maximum values of iron, copper and zinc than those grown in Aquil (Table 5.4): 11.91 vs. 0.30 ug-at per g dry wt. Fe, 1.08 vs. 0.30 ug-at per g dry wt. Cu, and 2.92 vs. 0.70 ug-at per g dry wt. Zn. Tissue manganese concentrations ranged from not detectable to 0.46 ug-at per g dry wt. for Aquil juveniles, but were not detected in oceanic kelp.

5.3.12 Comparison of Aquil Cultured and Seawater-Tray Cultured Juveniles

Elemental tissue concentrations of three rope culture juvenile plants were determined by XRF at Crocker Nuclear Laboratory, University of California, Davis in October, 1976 (Table 5.8). Comparison with average elemental concentrations of juveniles grown in Aquil (Table 5.5) showed distinct differences. Iron was two to four times higher, zinc was five to seven times higher, and copper was two times lower than for Aquil-cultured juveniles. Manganese levels were comparable.

Arsenic was much higher in seawater tray plants (0.47 - 0.55 ug-at per g dry wt.) than for Aquil-cultured plants (0.04 ug-at per g dry wt.). Vanadium, chromium, nickel and cobalt concentrations were comparable, while molybdenum was five times lower in seawater-tray plants.

Important differences existed between the conditions for juveniles cultured in Aquil and those grown in seawater. Aquil-culturing was a constrained system, consisting of defined amounts of completely dissolved ions, guaranteed by the presence of the strong chelator EDTA. Ocean water, however, was an unconstrained system. Precipitates (especially of  $\text{Fe}(\text{OH})_3$ ) probably led to adsorption/desorption of other ions, and juveniles were exposed to fluctuating micronutrient levels as well as organics released from other biota. Kelp could possess large internal stores of metals, absorbing strongly from

Table 5.8 Elemental tissue concentrations determined by XRF for Macrocystis juveniles grown in running filtered seawater from Newport Bay, at the Kerckhoff Marine Lab. Plants came from Rope Culture 1.

<u>Plant No.</u>	<u>Tissue Concentration,</u> <u>ug-at/g dry wt.</u>			
	<u>Mn</u>	<u>Fe</u>	<u>Cu</u>	<u>Zn</u>
NBS <sub>1</sub>	.22	4.46	<.08	2.80
NBS <sub>2</sub>	.22	3.13	<.06	2.40
NBS <sub>3</sub>	.23	2.61	<.06	1.87
Range	.22- .23	2.61- 4.46	<.06- <.08	1.87 2.80

<u>Element</u>	<u>Range, ug-at/g dry wt.</u>
As	.47 - .55
Cr	<.27 - <.31
Mo	<.06 - <.07
Ni	<.07 - --
Pb	.021 - .023
V	<.45 - .58
Co	<.08 - <.10

seawater when levels rose to high concentrations.

#### 5.4 Background Levels of Metals

The air system, media, and Q-water were examined as possible sources of contamination to explain why the XRF levels in Macrocystis did not reflect differences in the Aquil formulations.

##### 5.4.1 Air Supply Background Levels

The laboratory air supply was filtered through a 0.20 um all-plastic Gellman air filter as described previously (Section 2.4.4, Figure 2.5) before bubbling through the media at 1 liter per minute.

Recent work by Ouimette (1981) determined the total aerosol species based on diameter range. A single sample was taken on the roof of Guggenheim Laboratory at Caltech, from 1127 to 1431 PST on February 6, 1980. The aerosol was size segregated by low pressure impactor (LPI) onto vaseline-coated mylar and analyzed by particle induced x-ray emission (PIXE) at Crocker Nuclear Laboratory, University of California, Davis. This sample is estimated to represent a day in the upper 90th percentile of highly polluted days in Los Angeles (J. R. Ouimette, personal communication).

Although the Gellman air filter removes particles down to 0.20 um, the stages of the LPI overlap this size. The aerosol concentrations of Fe, Mn, Cu, Zn and Pb in the three lowest stages (from 0.05 to 0.26 um) were calculated and summed (Table 5.9). No other micronutrients in Aquil were determined. Fe, Zn and Pb showed the highest values, ranging

from  $1.43$  to  $3.84 \times 10^{-12}$  moles per liter of air, followed by Cu at  $1.60 \times 10^{-13}$  moles per liter of air. Mn was not detected. Fe, Mn, Cu, Zn and Pb have detection limits of  $8.5 \times 10^{-14}$ ,  $8.6 \times 10^{-14}$ ,  $5.8 \times 10^{-14}$ ,  $8.0 \times 10^{-14}$ , and  $8.9 \times 10^{-14}$  moles per liter of air, respectively.

The theoretical metal contamination from air to media was calculated from Ouimette's data assuming that the sample of aerosol was representative of the laboratory air supply and that there was 100% transfer efficiency from air through the filter to Aquil media (Table 5.10). The maximum concentrations would have occurred at three day intervals, when the media were replaced. Fe, Cu and Zn experiments would have been affected only at the lower end of the concentration range tested. The concentration of Mn was taken as the detection limit,  $< 8.6 \times 10^{-14}$  moles per liter of air. Mn experiments would not have been affected, except possibly for the 0 nM.

#### 5.4.2 Air, Q-water and Media Background Metal Levels

The juvenile kelp culturing system was tested to examine background levels of Fe, Mn, Cu, and Zn. Six flasks of Aquil without added micronutrients were tested in the growth chamber for nine days. Filtered air at one liter per minute was passed through three of the Q-water flasks and all three Aquil flasks. Flasks were removed from the chamber every three days when bubblers were removed and then reassembled, imitating the media replacement sequence of kelp culturing.

Table 5.9 Total elemental aerosol species in diameter range 0.05 to 0.26  $\mu\text{m}$ . Sample was obtained on roof of Guggenheim Laboratory, Caltech, 1127-1431 PST, February 6, 1980. Aerosol was size segregated by LPI onto vaseline coated mylar and analyzed by PIXE.

<u>Element</u>	<u>Concentration</u> <u>Moles/liter of air</u>
Fe	$2.14 \times 10^{-12}$
Mn	$< 8.6 \times 10^{-14}^*$
Cu	$1.60 \times 10^{-13}$
Zn	$1.43 \times 10^{-12}$
Pb	$3.84 \times 10^{-12}$

\* Detection limit =  $8.6 \times 10^{-14}$  moles Mn/liter of air.

Table 5.10 Comparison of theoretical metal contamination from air and various Aquil formulations.

<u>Element</u>	<u>Metal in Air, from 3 day Supply of Air to .9 liter of Aquil, nM</u>	<u>Metal Concentration in Various Aquil Formulations, nM</u>
Fe	11.5	0, 10, 250, 1000
Mn	< 0.4 <sup>*</sup>	0, 5, 25, 100
Cu	.77	0, 2.5, 5, 10, 50
Zn	6.9	0, 2, 10, 200, 1000
Pb	18.43	Not Added

\*This value corresponds to detection limit in air.

Samples from the nine flasks were acidified with 1% Ultrex nitric acid and stored in the dark in the refrigerator. Atomic absorption spectrophotometry (AAS) analysis was performed on these samples with a Varian Techtron AAS Model #AA6. Fe, Mn, Zn and Cu were not detected in any of the flasks. Detection limits for the metals were 17 nM Fe, 18 nM Mn, 7 nM Cu, and 15 nM Zn. Unfortunately, these values are higher than values given in Table 5.10.

Other investigators have considered possible contamination levels in Q-water and Aquil in the absence of bubbling with air. Two metal levels, one for Cu and one for Zn, are available for comparison. Steven H. Lieberman used AAS analysis to test for the presence of Cu in Q-water used for Kuwabara's Aquil experiments (personal communication). Using clean techniques, Cu was not seen above a detection limit of 1 nM. Anderson, Morel and Guillard (1978) deduced from calculations that the background level of Zn in Aquil formulations for coastal diatoms was less than 5 nM Zn.

### 5.5 Mass Balance Calculations

Mass balance calculations examined two possible reasons why micronutrient levels in plants did not reflect differences in the Aquil formulations: A. juveniles instead used micronutrients already stored in the tissues, and thereby "diluted" the tissue concentration levels; or B. levels of contamination in air, Q-water or media provided a source of micronutrients. Finally, using lead data, the possibility of air as a source of contamination is assessed.



Mass balance calculations were done using XRF data on plants grown in Aquil deficient in micronutrients, including three plants each from Series #20 through #50 and all 21 plants from Series #90.

#### 5.5.1 Mass Balance Calculations for Dilution Effects

To examine whether a dilution effect occurred, total initial moles of Fe, Mn, Cu and Zn in each juvenile blade were compared with the final total moles for metal deficiency experiments. Elemental tissue concentrations determined by XRF for juveniles grown in the seawater tray were used for the initial concentrations of the test plants (Table 5.8). XRF analysis gave the final elemental tissue concentration in each plant at the end of the experiment.

The increase or decrease in the total number of moles of each metal in one flask at the end of the experiments was determined as  $\Delta$ ,  $(N_f W_f - N_i W_i)$ .  $\Delta$  is calculated for metal deficiency experiments Series #20 through 50 and Series #90 in Tables 5.11 and 5.12, respectively.

Iron increased by an average factor of 1.4, Mn by 29, Cu by 15 and two Zn plants by 1.4 and 2.0. The other four Zn plants decreased by an average factor of 1.8. This suggested that the Zn-deficient plants were candidates for dilution effects. In nutrient deficiency experiment Series #90, 11 of the 12 plants showed an increase in the total number of moles of Fe, Mn, Cu and Zn. Only one plant, (Fe<sub>1a</sub>), showed a decrease by a factor of 2. The other two Fe plants increased by factors

Table 5.11 Mass balance calculation for micronutrient deficiency tests in individual series #20 to #50. Initial and final weights of the juveniles,  $W_i$  and  $W_f$ , respectively were measured. The final tissue elemental concentration,  $N_f$  for the test plants was determined by XRF. Initial tissue metal concentration averages for three juvenile plants was used for  $N_i$ , the initial tissue metal concentration. A conversion factor of 0.1 g dry wt. to 1.0 g wet wt. was used for juvenile sporophytes.

Series No.	Deficient Element in Media, Plant No.	Deficient Element, ug-at/g dry wt.		Wet Weight of Juveniles, mg		Total Moles ( $\times 10^{10}$ )		$\Delta$ , Moles ( $\times 10^{10}$ )
		$N_i$	$N_f$	$W_i$	$W_f$	$N_i W_i$	$N_f W_f$	
		Tissue Elemental Concentration of						
20	Fe <sub>1</sub> a	3.4	0.57	7	77	24.	44.	31.0
	b	3.4	0.46	7	65	24.	30.	
	c	3.4	0.54	7	54	24.	29.	
30	Mn <sub>3</sub> a	.22	.19	5.3	73	1.2	14	79.4
	b	.22	.16	5.3	204	1.2	33	
	c	.22	.12	5.6	298	1.2	36	
	Mn <sub>4</sub> a	.22	.16	4.5	123	1.0	20	116.6
	b	.22	.18	5.4	154	1.2	28	
	c	.22	.25	5.6	287	1.2	72	
40	Cu <sub>1</sub> a	---	---	4.7	---	---	---	---
	b	---	---	4.9	---	---	---	
	c	---	---	5.0	---	---	---	
	Cu <sub>2</sub> a	.07	.11	5.1	38.7	.36	4.3	15.0
	b	.07	.11	5.0	54.4	.35	5.6	
	c	.07	.11e	5.3	55.9	.37	6.2	
50	Zn <sub>1</sub> a	2.36	.50	4.9	11.4	11.6	5.7	2.4
	b	2.36	.50e	5.0	11.7	11.8	5.9	
	c	2.36	.50e	5.3	53.3	12.5	26.7	
	Zn <sub>2</sub> a	2.36	.23	4.7	28.7	11.1	6.6	-4.3
	b	2.36	.23	4.9	31.7	11.6	7.3	
	c	2.36	.42	5.3	39.5	12.5	17.0	

e = estimated as average of values of other plants in the same flask.

$$\Delta = (N_f W_f - N_i W_i)$$

Table 5.12 Mass balance calculation on micronutrient deficiency experiment series #90. Initial and final weights of the juveniles,  $W_i$  and  $W_f$ , respectively were measured. The final tissue elemental concentration,  $N_f$  for the test plants was determined by XRF. Initial tissue metal concentration averages for three juvenile plants was used for  $N_i$ , the initial tissue metal concentration. A conversion factor of 0.1 g dry wt. to 1.0 g wet wt. was used for juvenile sporophytes.

Deficient Element in Media, Plant No.	Tissue Elemental Concentration of Deficient Element ug-at/g dry wt.		Wet Weight of juveniles, mg		Total Moles ( $\times 10^4$ )		$\Delta$ , Moles ( $\times 10^{10}$ )	
	$N_i$	$N_f$	$W_i$	$W_f$	$N_i W_i$	$N_f W_f$		
Fe <sub>1</sub>	a	3.40	.88	4.5	8.0	15.3	7.0	175.5
	b	3.40	.78	4.5	123.5	15.3	96.3	
	c	3.40	.83e	4.5	142.3	15.3	118.1	
Mn	a	.22	.41	4.5	111.2	1.0	45.6	105.0
	b	.22	.24	4.5	140.4	1.0	33.7	
	c	.22	.19	4.8	151.8	1.1	28.8	
Cu	a	.07	.21	4.5	140	.31	29.4	68.7
	b	.07	.19	4.9	152	.34	28.9	
	c	.07	.07	5.2	162.6	.36	11.4	
Zn	a	2.36	.24	5.0	93.2	11.8	22.4	82.5
	b	2.36	.22	5.0	111.2	11.8	24.5	
	c	2.36	.61	5.0	116.4	11.8	71.	

e = estimated as average of values of other plants in the same flask.

$$\Delta = (N_f W_f - N_i W_i)$$

of 6 and 8. Manganese plants increased by an average factor of 35, Cu by 71 and Zn by 3. So except for plant Fe<sub>1a</sub>, a dilution effect could not be considered for Series #90.

#### 5.5.2. Mass Balance Calculation for Air or Media Contamination

Contamination levels necessary in air and media to account for XRF tissue levels in the micronutrient deficiency experiments were calculated for Series #20 through #50 (Table 5.13) and Series #90 (Table 5.14). The worst case was again considered, using 100% transfer efficiency for metal through the air filter, 100% removal of the metal by the media from the air bubbles, and 100% absorption efficiency from media to plants.

Concentrations required in the air ranged from  $2.4 \times 10^{-13}$  moles Fe per liter air to  $9.0 \times 10^{-13}$  moles Mn per liter air for Series #20 through #50. Both flasks of Zn showed a decrease in total moles of Zn, requiring no outside source. Series #90 needed a higher range from  $5.3 \times 10^{-13}$  moles Cu per liter of air to  $13.5 \times 10^{-13}$  moles Fe per per liter of air. However, the total elemental aerosol calculated from Ouimette's data (Table 5.9) met or exceeded the air levels of Fe, Cu and Zn necessary for Series #20 through #50 and Fe and Zn for Series #90.

Cu in the air is about a factor of two too low to account for Series #90 Cu increases. The concentration of Mn taken as the detection limit,  $< 8.6 \times 10^{-14}$  moles of Mn per liter air, cannot meet the

Table 5.13 Contamination levels necessary in air or media to account for tissue elemental levels determined by XRF analysis for series #20 through #50. A 100% absorption efficiency from air through filter to media to plants is assumed.

Series No.	Flask No.	Deficient Element	$\Delta$ , Moles ( $\times 10^{10}$ )	Contamination Level Necessary to Meet XRF Levels ( $\Delta$ /volume of air or media)	
				Air ( $\times 10^{13}$ Moles/l)	Media (nMoles/l)
20	Fe <sub>1</sub>	Fe	31.0	2.4	1.2
30	Mn <sub>3</sub>	Mn	79.4	6.1	2.9
	Mn <sub>4</sub>	Mn	116.6	9.0	4.3
40	Cu <sub>2</sub>	Cu	15.0	1.2	0.6
50	Zn <sub>1</sub>	Zn	+2.4	0.2	0.1
	Zn <sub>2</sub>	Zn	-4.3	0	0

Table 5.14 Contamination levels necessary in air or media to account for tissue elemental levels determined by XRF analysis for series #90, micronutrient deficiency experiment. A 100% absorption efficiency from air through filter to media to plants is assumed.

Flask No.	Deficient Element	$\Delta$ , moles ( $\times 10^{16}$ )	Contamination Level Necessary to Meet XRF Levels ( $\Delta$ /volume of air or media)	
			Air ( $\times 10^{13}$ Moles/l)	Media (nMoles/l)
Fe <sub>1</sub>	Fe	175.5	13.5	6.5
Mn <sub>2</sub>	Mn	105.0	8.1	3.9
Cu <sub>3</sub>	Cu	68.7	5.3	2.5
Zn <sub>4</sub>	Zn	82.5	6.4	3.1

requirements shown by XRF data:  $6.1 \times 10^{-13}$ ,  $9.0 \times 10^{-13}$  and  $8.1 \times 10^{-13}$  M for Series #30, #40 and #90, respectively.

According to mass balance calculations shown in Table 5.10, if the calculated airborne contamination levels existed, several Aquil experiments would be brought into question. In particular these are 0 and 10 nM Fe, 0 nM Mn, 0 nM Cu, and 0, 2 and 10 nM Zn.

### 5.5.3 Lead as an Indicator

Due to the lack of conclusive evidence from direct mass balance calculations, Pb may instead be considered as an indicator. A high lead concentration of  $3.84 \times 10^{-12}$  moles per liter of air was detected in the Pasadena air. Work by Manley (personal communication) has shown that Pb is actively taken up by kelp blades, and displaces metals. If Pb occurred at high levels in the air supply, and air contamination was occurring, juveniles would show high levels of lead when compared to the background levels represented by the seawater tray plants.

An exposure of the plants over three days to air contaminated by metals at levels predicted by Ouimette's data would result in a media level of 18.43 nM Pb (Table 5.10).

Seawater tray plants showed 0.021 to 0.023 ug-at per g dry wt. Pb (Table 5.8) and the average elemental concentration in Aquil-cultured juveniles was only 0.04 ug-at per g dry wt. Pb (Table 5.5). Theoretically, the air supply did not contribute to metal contamination

of the Aquil system.

The very low levels of Pb in XRF analyses indicate that the amounts of the other elements that were detected could not have been of airborne origin. Furthermore, absence of Mn at detectable levels in the air but its presence in the final dry tissues suggests a non-atmospheric source must have existed.

It is therefore believed that the plants were either contaminated during sample preparation at the Kerckhoff Marine Laboratory prior to analysis, or while undergoing XRF analysis, or the changes in the metabolically-active fraction within the kelp tissues were too low to be detected by XRF analysis.

#### 5.6 Macrocystis - Chelator Model

Research by Kuwabara (1980) has shown that the gametophytic form of Macrocystis is highly sensitive to the nanomolar micronutrient levels in Aquil that had little effect on juvenile Macrocystis sporophytes. Optimal micronutrient levels for Aquil gametophytes were lower than the maximum levels (also non-toxic) tested on Aquil sporophytes (Table 5.15). Toxicity of Fe, Mn, Cu, Zn and Co produced dramatic reduction in growth in gametophytes, and a deficiency of these micronutrients resulted in reduced growth rates or death.

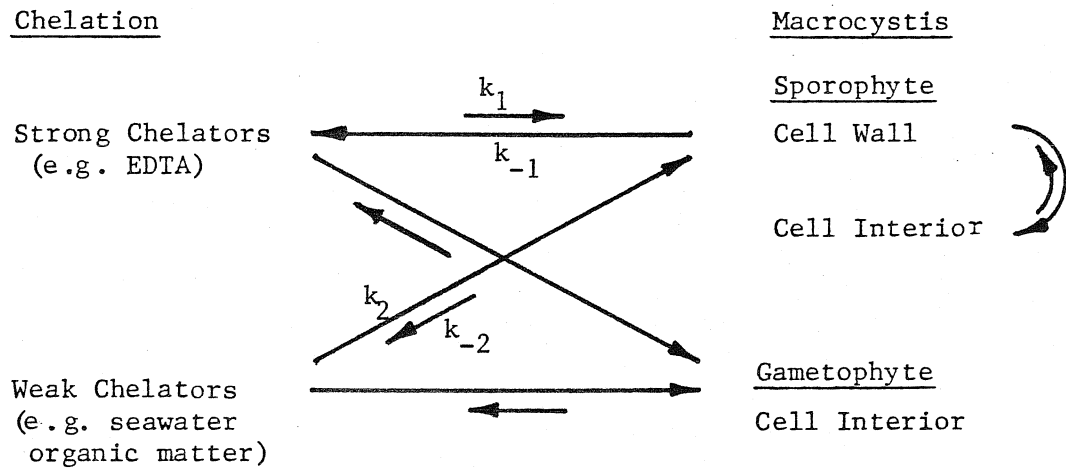
It is possible that different types of pathways for micronutrient uptake exist. Movement of a metal from a bulk phase into a cell interior consists of transport steps linked with chemical reactions.



Table 5.15 Optimal and toxic Aquil micronutrient levels for Macrocystis gametophytes compared with the highest levels tested (also non-toxic) for Macrocystis sporophytes.

<u>Element</u>	<u>Micronutrient Concentration, nM</u>		
	<u>Gametophyte</u>		<u>Sporophyte</u>
	<u>Optimal</u>	<u>Toxic</u>	Maximum Concentration Tested, (Non-toxic)
Fe	350	1000	1000
Mn	30	32	100
Cu	10	15	500
Zn	170	300	1000
Co	70	100	300
Mo	100	-	1000
I	100	-	500

Figure 5.1 Model for micronutrient uptake from Aquil by juvenile Macrocystis. Length of arrows is proportional to the theoretical transport.



However, if the overall product is kinetically dominated by one slow reaction, then this pathway can be envisioned as a single-step process. This single-step process for uptake by gametophytes is represented as a two-step process: first from the bulk solution to the cell wall, and second from the cell wall to the cell interior. The dominant relative rate is designated by the size of the arrow.

In natural seawater, weak chelators in the form of organic matter may exist as well as strong chelators that are exuded from organisms. In Aquil, the strong chelator EDTA dominates the media chemistry of the micronutrients. Wheeler (1979) showed that radiolabeled EDTA is not taken up by adult Macrocystis blades, and therefore EDTA provides strong competition for the plant in obtaining micronutrients.

Electron microscopy studies of Macrocystis pyrifera (Gherardini and North, 1971) showed that the cell wall of the gametophytes was about one-tenth the thickness of that of the early sporophytes (nine-cell). Research by Gherardini and North was confirmed in subsequent work by Chen using small sporophytes (personal communication).

Uptake for a gametophyte may be a single-step process, as previously described. This would explain why the growth among Aquil-cultured gametophytes was highly responsive to changes in micronutrient concentrations. The thick cell wall of the sporophyte may act as buffer for high external concentrations or as storage for micronutrients. It may be a rate-limiting step for the micronutrient to

be absorbed from the media to the cell wall. Thus the growth among *Aquile* sporophytes was not responsive to changes in micronutrient concentrations.

The dynamics of exchange of metals from chelates in the case of juvenile kelp needs to be considered. How easily the metal is transferred is a critical factor. A slow exchange rate would mean the form of the metal unimportant to the kelp. Thus, the forward relative rate constant,  $k_1$  (Figure 5.1) would be dominated by the backward rate constant,  $k_{-1}$ . A slow exchange rate is characteristic of the metal-EDTA chelate combinations found in *Aquile*. Juvenile kelp were insensitive to a change of free metal ion concentrations of one order of magnitude or more.

If rapid exchange rates characterize chelates formed from metal and organic matter occurring in seawater, then the forward relative rate constant  $k_2$  would dominate the backward rate constant,  $k_{-2}$  (Figure 5.1). Speciation would thus be important to juvenile kelp grown in seawater. As described previously, North (1980) found a much higher sensitivity for juveniles to metal additions in seawater.

Why the sporophytes were unaffected by lack of a micronutrient during nine-day-duration experiments needs to be examined. The sporophytic requirements for micronutrients may be much less than the micronutrients available. A small change in the tissue elemental concentrations could never be detected by XRF analysis. Juvenile

sporophytes eventually degraded when they were Aquil-grown for 32 days in Series #90, indicating that they can survive for a long period but not without limits when a micronutrient is lacking in Aquil.

Gametophytes are highly responsive to changes in micronutrient concentrations in Aquil, and experimental concentrations for this work were selected on this basis. For future work, concentration ranges should be even greater than the 100-fold increase over Kuwabara's (1980) ranges used in this research. Additionally, the duration of the experiments should be longer.

Tissue concentrations of Cu and Zn did not rise in Aquil sporophytes as they did in seawater sporophytes when Cu and Zn concentrations in the medium were raised. However, comparison between artificial media and natural seawater can only be done with caution, as previously discussed in Section 5.3.12. Possibly an addition of Cu in the Aquil-EDTA system does not increase the free cuprous ion ( $\text{Cu}^{2+}$ ) concentration as much as it does when it is added to seawater. EDTA may buffer the toxic effects. Aquil experiments were also started at much lower levels of Cu and Zn than North's work (1980).

#### 5.7 Comparison of Aquil and Ocean Water Limiting Concentrations

Micronutrient experiments with Aquil formulations show that juvenile Macrocystis flourish at nanomolar levels of micronutrients. JWPCP effluent from the Los Angeles County Sanitation Districts passes through an outfall system at Whites Point to be diluted with ocean

water. Ocean water limiting concentrations, established by the California State Water Resources Control Board, were met for elements listed in Table 5.17, assuming a 100-fold dilution of mean effluent with seawater (Table 5.16).

The level of iron in diluted effluent was approximately three times as large as the corresponding level in Aquil SB, and copper was six times as large (Table 5.17). However, juveniles grew well in Aquil with 500 nM copper. Diluted effluent levels of manganese were 10 times below the Aquil level that induced a toxic response in the juvenile kelp. Effluent values for zinc, cobalt and molybdenum were about equivalent, 25 and 30 times smaller, respectively.

Table 5.16 Section A shows ocean water limiting concentrations (State of California, California State Water Resources Control Board, 1978). Section B shows 1980 JWPCP mean effluent composition (County Sanitation Districts of L.A. County, 1981).

<u>Element</u>	A Ocean Water <u>Limiting Concentrations, nM</u>		B JWPCP <u>Effluent Composition, nM</u>
	<u>6-month Median</u>	<u>Daily Maximum</u>	
Fe	--	--	71,087.
Mn	--	--	1456.
Cu	79.	314.	2990.
Zn	306.	1224.	9178.
Co	--	--	441.
Mo	--	--	313.
I	--	--	--
As	107.	427.	66.
Cd	27.	107.	178.
Cr	39.	154.	5961.
Ni	340.	1362.	3406.
Hg	0.7	2.8	4.
Pb	39.	154.	58.
Ag	4.2	17.	0.9

Table 5.17 Comparison of micronutrient concentrations in the synthetic seawater Aquil SB and 100-fold diluted JWPCP outfall effluent.

<u>Element</u>	<u>Aquil SB, nM</u>	<u>Diluted JWPCP Effluent, nM</u>
Fe	250.	710.
Mn	10.	14.
Cu	5.	30.
Zn	100.	91.
Co	100.	4.
Mo	100.	3.



## Chapter 6

## CONCLUSIONS

6.1 Iron Reduction Experiments

1. Juvenile Macrocystis plants reduce Fe(III) to Fe(II), as do terrestrial plants.
2. Light alone reduced Fe(III) to Fe(II), but not as rapidly as kelp.
3. Kelp reduction of Fe(III) was enhanced by light.
4. The preceding conclusions support the hypothesis that Fe(III)EDTA is reduced and then dissociates external to the plant.
5. Based on tissue iron concentrations determined by XRF, in the presence of BPDS, juveniles reduced about 14 times the amount of iron needed for new biomass produced.
6. The rate of Fe(III) reduction per unit surface area for juvenile Macrocystis, ( $9.14 \times 10^{-11}$  moles Fe(III) per  $\text{cm}^2$  per day) was the same order of magnitude as that shown by Owens and Chaney (1971) for the fresh water green alga Chlorella sorokiniana.

## 6.2 Micronutrient Experiments

7. Growth rates up to 0.38 per day were measured for juvenile Macrocystis grown in Aquil.

8. Kelp juveniles grew fastest with 250 nM Fe, 5 nM Mn, 1000 nM Zn, 1000 nM Mo and 300 nM Co. Concentrations of iodine up to 500 nM I<sup>-</sup> and copper up to 500 nM produced neither stimulatory nor inhibitory responses.

9. Aquil concentrations of 500 nM Fe, 500 nM Cu, and below 1000 nM Zn were in the kelp non-interactive category, i.e. increased medium concentration did not produce increased tissue concentration. 1000 nM Zn was in the interactive category, where tissue zinc levels increased. Manganese was always in the non-interactive category up to and including 100 nM.

10. Iron deficiency was associated with elevated concentrations of zinc in the tissues.

11. The culturing system was operated for nine days without any kelp plants and without added micronutrients to check against possible contamination of the air system, Q-water, or media by trace metals. AAS analysis failed to reveal any contamination, although detection limits by this method were too high to be conclusive in ruling out air as possible source of contamination. Estimates of mass fluxes of airborne contaminants based on Ouimette's data also were high enough so that the

air could not be ruled out as a possible contamination source of Fe, Mn, Cu and Zn.

12. Mass balance calculations for nutrient deficiency experiments revealed that total amounts of Fe, Mn, some Cu and some Zn in Aquil-cultured kelp tissues increased. This suggests that a contamination source must have existed for some cases, i.e. when the Aquil design level was zero. There was no method to prove that air contamination of the media at such low metal levels did occur. However, absence of high Pb concentrations in the tissues indicated that the air supply during culturing was not contaminating the media. An absence of Mn at detectable levels in the air but its presence in dry kelp tissues suggests that a non-atmospheric source must have existed. Contamination may have occurred during processing tissues for XRF.

13. A Macrocystis-Chelator model was proposed to explain results obtained from experiments described herein and differences observed between sensitivities of gametophytes and sporophytes to elevated concentrations of trace metals. The model postulates an important role by the cell wall in adsorption metals.

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