MICRONUTRIENT REQUIREMENTS FOR *MACROCYSTIS PYRIFERA* (L.) C. A. AGARDH (GIANT KELP) GAMETOPHYTES DETERMINED BY MEANS OF A CHEMICALLY DEFINED MEDIUM, AQUIL

Thesis by

James S. Kuwabara

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1980

(Submitted April 30, 1980)

ACKNOWLEDGMENTS

Thanks are extended to Professor Wheeler J. North for research supervision. His encouragement, patience and insight into biological systems was inspiring. Professor James J. Morgan provided advice on chemical aspects. Cooperation and interactions from other Caltech faculty were deeply appreciated.

Editorial assistance from Dr. James F. Pankow, Oregon Graduate Center, was invaluable. Discussions on research problems with Drs. Gregory Gartrell, Jr., Valrie A. Gerard, Stephen H. Lieberman, Steven L. Manley and Francois M. M. Morel, Massachusetts Institute of Technology, were invariably productive.

Elton F. Daly, Joseph J. Fontana and Richard Eastvedt assisted in fabricating culturing apparatus. Contributions by the following are also acknowledged with appreciation: Barbara B. Barth for editing this thesis; Laurence G. Jones for preparation of drawings; Peter A. Allison, Randall W. Berthold, Henry C. Fastenau, William Smith and Thomas G. Stephan for assistance in sporophyll collection; and Windsor Sung for his insight into redox and adsorption processes.

Financial support by the National Science Foundation, the Department of Energy, the Department of Commerce, Office of Sea Grants, Kelco Co. and Union Oil Co. is gratefully acknowledged.

Special thanks go to Ann L. Long for typing and editing. Her advice on Macrocystis gametophytic culturing was immensely helpful.

ABSTRACT

Requirements of Macrocystis pyrifera (L.) C. A. Agardh gametophytes for nine nutrients (Co, Cu, Fe, I, Mn, Mo, N, P and Zn) were established by means of the chemically defined, artificial seawater, Aquil. Optimal growth ranges for total concentrations of Co, Cu, Fe, Mn, Zn and NO_3-N and for media temperatures were determined. Effects on Macrocystis gametophytic growth within these optimal ranges were then investigated. Mathematical equations modeling gametophytic length as a function of concentrations of NO_3-N, Fe and Zn, and Zn, Mn, Cu and Co were generated by least squares fit of experimental data.

An Aquil formulation was developed that consistently produced Macrocystis embryonic sporophytes after 12 culturing days. Sporophyte production was achieved in Aquil using sporophylls from a variety of southern California sampling sites to initiate gametophytic cultures. This marks the first instance that any macroalga has been successfully cultured in a chemically defined artificial seawater medium using ultraclean techniques.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONT.)

TABLE OF CONTENTS (CONT.)

LIST OF FIGURES

LIST OF FIGURES (cont'd)

LIST OF TABLES

LIST OF TABLES (cont'd)

LIST OF TABLES (cont'd)

LIST OF TABLES (cont'd)

LIST OF TERMS AND ABBREVIATIONS

EDTA	ethylenediaminetetraacetic acid (also disodium edetate)
FEP	fluoroethylene polymer
KMLSW	1:1 mixture of surface and 300 m seawater sampled from a site (referred to as the Triangle Station) 6 miles south of Newport Bay
l_c	cell length
LPE	linear polyethylene
MBSW	unenriched Monterey Bay surface seawater
[Me]	molar concentration of a metal Me
Me_T	total concentration of a metal Me (e.g., Cu_T = total copper concentration
n$\underline{M}$	10^{-9} moles/liter (i.e., nanomolar concentration)
p units	-log concentration units (e.g., $pCu_T = 8$ implies $Cu_T = 10^{-8}$ $\underline{M}$)
R_c	cell radius
TFE	tetrafluoroethylene
total concentration	analytical concentration
x_i	modeling variable transformed to normalize an experimental variable (i.e., $\|x_i\| < 1$). For example, if x_i represents Cu_T within the range [10 n$\underline{M}$, 50 n$\underline{M}$], then $x_i = 0.5$ corresponds to $Cu_T = \frac{(50-10)}{2} + \{(+0.5)\frac{(50-10)}{2}\}$ = 30 n$\underline{M}$
μ	10^{-6} meter (i.e., micron)
$\mu\underline{M}$	10^{-6} $\underline{M}$ (i.e., micromolar concentration)

CHAPTER 1

INTRODUCTION

Project Selection.

Macrocystis pyrifera (L.) C.A. Agardh (giant kelp) is an ecologically and commercially significant brown algal species of the western coast of North America and of every major land mass in the temperate southern hemisphere. Adult plants form dense beds frequently extending many kilometers along the coast. Kelp fronds provide both a settling substrate and a shelter for many benthic organisms such as crustaceans, mollusks and bryozoans. In addition, *Macrocystis* is eaten by grazing fish such as opaleye, *Girella nigricans* and halfmoon, *Medialuna californiensis*, urchins such as *Strongylocentrotus franciscanus* and *S. purpuratus*, and many other marine animals. Kelp beds may also present a physical barrier against storm swells.

The major commercial use of *Macrocystis* is presently for alginic acid production. However, increasing scarcity of fossil fuels has prompted a growing interest in *Macrocystis* as a fuel source via bioconversion of solar energy. Under laboratory conditions, kelp growth rates sometimes exceed 30% per day on a wet weight basis (North, 1978a). Researchers at Kerckhoff Marine Laboratory are examining the feasability of growing adult kelp plants on artificial substrates moored in the open ocean.

Surface waters of the open ocean are typically nutrient depleted. Nutrient-rich water from several hundred meters below the surface is pumped up and dispersed around these plants. We have no assurance that deep water represents an optimal fertilizing medium for kelp. An understanding of the nutrient requirements for Macrocystis is therefore important for optimizing our fertilizing system.

Growth rates of organisms may vary according to environmental conditions. Effects of temperature and light intensity on Macrocystis gametophytes have been examined by Lüning and Neushul (1978). Jackson (1977), and North and Wheeler (1977) have studied influences of nitrogen and phosphorus concentrations on the growth of adult and juvenile Macrocystis plants. The essential nature of micronutrients for Macrocystis juvenile growth are presently being examined in separate studies.

This thesis examines trace element requirements (Co, Cu, Fe, Mn, Zn) of Macrocystis. Experimentation fell into three categories. Using Aquil as our basal culturing medium (see Chapter 3), we first developed a method for producing a chemically defined seawater medium that sustained gametogenesis and sporophyte production. This research phase lasted 14 months and required ten sequential experiments. Results from each experiment were analyzed to prescribe necessary changes in variable levels to improve gametophytic growth in the succeeding series of experiments. The second

phase of experimentation established the essential character of each nutrient variable (Co, Cu, Fe, I, Mo, Mn, N, P, Zn) present in the successful Aquil formulation. Primary and interactive micronutrient effects on gametophytic growth were then investigated within the optimal concentration ranges determined for Co, Cu, Fe, Mn, N and Zn in the first experimental phase (Figure 1.1).

Life Cycle of Macrocystis.

Macrocystis is a marine macroalga which dominates many sublittoral communities of the southern California coast. It is taxonomically classified under Phylum Phaeophyta, Order Laminariales. The life cycle of Macrocystis involves an alternation of heteromorphic generations (Figure 1.2). With the exception of the embryonic stages, the sporophytic phase is macroscopic (adult plants can exceed 50 m in length). Fertile blades called sporophylls are basally located around the adult plant. These leaf-like structures annually release trillions of biflagellated spores. These haploid spores settle and adhere to a substrate. Gametophytic development ensues, culminating in gamete production. Oogonia on female gametophytes are fertilized by sperm cells from male gametophytes, yielding a diploid embryonic sporophyte. Successive mitotic divisions by sporophytic cells ultimately result in a macroscopic plant. The biology of the adult plant has been discussed in detail by North (1971).

Figure 1.1. Flow chart showing relations between the research phases.

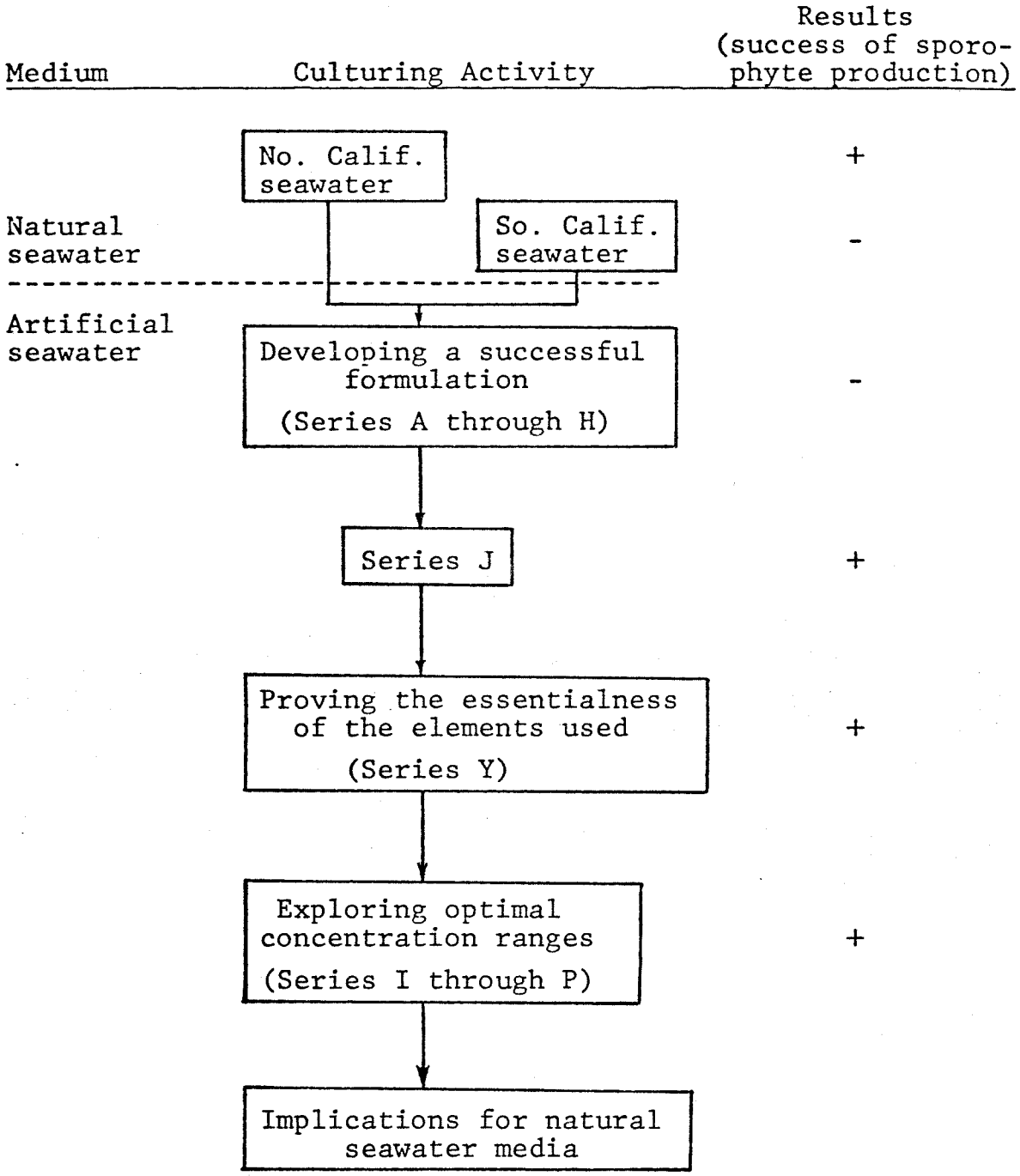
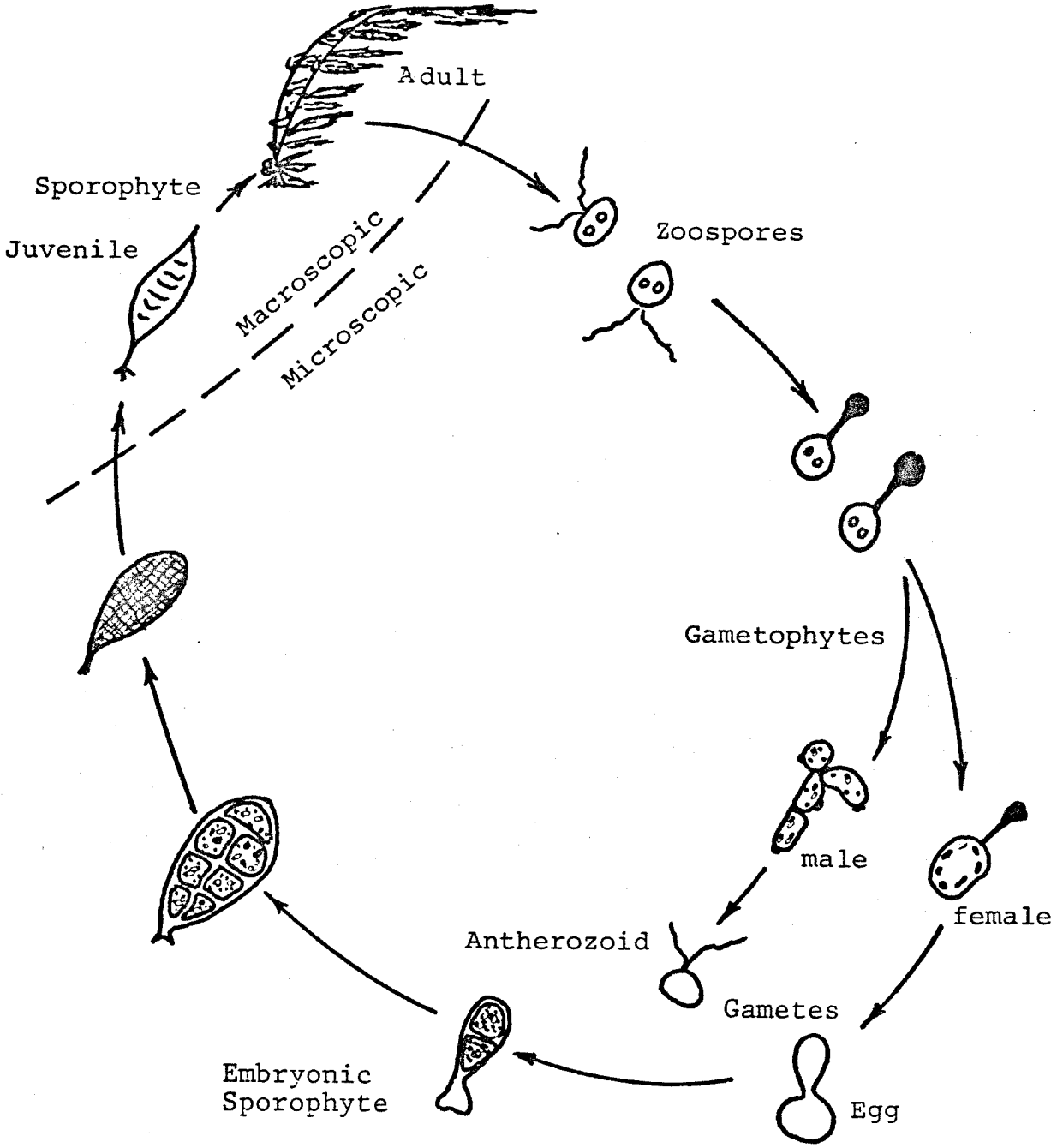


Figure 1.2. Life cycle of Macrocystis pyrifera (L.) C. Agardh (giant kelp)*.



*after North, 1971.

This thesis is concerned with the gametophytic phase of the Macrocystis life cycle and production of embryonic sporophytes. These stages were selected for study because 1) gametogenesis and sporophyte production, the natural outcome of the gametophytic phase, provide an unequivocal biological criterion for assessing suitability of growth media; 2) microscopic size of gametophytes facilitated construction and operation of an ultraclean culturing system; and 3) their relatively short duration permitted completion of the many sequential experiments needed for a complete evaluation within a reasonable time frame.

CHAPTER 2

EXPERIMENTAL VARIABLES

Introduction.

As early as 1895, work by Mölisch revealed the necessity of certain mineral salts for successful algal culturing. Recent advances in mathematical and chemical analysis techniques have greatly enhanced possibilities for determination of plant nutrient requirements.

Research in algal nutrition has shown that: 1) numerous elements are required, some at very low levels, 2) mineral requirements differ among species, 3) amounts of each required element may differ both between and within algal species under different culturing conditions, and 4) certain trace nutrients may be essential for specific metabolic reactions.

Requirements for the micronutrients, cobalt, copper, iron, manganese and zinc by Macrocystis pyrifera gametophytes for growth and development were explored. Various levels of the macronutrient nitrate as well as water temperature were also examined for comparison with trace element effects.

Each of the seven experimental variables selected for this study is briefly discussed below:

Cobalt.

Data by Yamamoto (1965) and North (1977a) have demonstrated that brown algae concentrate cobalt. Cobalt was found to affect photosynthesis of Chlorella pyrenoidosa (Emerson and Lewis, 1939). Hutner et al. (1958) discovered cobalt in the molecular structure of vitamin B-12 cyanocobalamine. However, Scott and Ericson (1955) found little relationship between cobalt fixation and vitamin B-12 synthesis in Rhodymenia palmata. The metabolism of cobalt remains a mystery.

Growth of a variety of blue-green algae and green algae have been stimulated by enriching culturing media with cobalt or vitamin B-12 (Holm-Hansen, 1954 and Krauss, 1955). Reported cobalt additions range from 20 nM (Toerien, 1971) to 4200 nM (Chu, 1942). Provasoli (1968) suggested the addition of $CoCl_2 \cdot 6H_20$ at a level of 620 nM. Thus, the range for prescribed levels of cobalt has been enormous. The same is true for other micronutrients. The variation of total cobalt (Co_T) values presents a problem for algal culturists because:

1. Different species of algae require cobalt in different chemical forms (e.g., some species fix cobalt for vitamin B-12 synthesis while others cannot).
2. Optimal cobalt addition to culturing media may depend on kinds and concentrations of other micronutrients (i.e., trace element interactions may exist).
3. Differences in culturing conditions such as pH,

light intensity, temperature, and concentrations of organic material will alter the chemical speciation of the medium's components as well as the metabolic rates of cultured organisms.

Copper.

Cytochrome-c oxidase, an enzyme involved with electron transfer in mitochondria of aerobic cells, contains two copper atoms. Other copper-containing oxidases include tryosinase, laccase and ascorbic acid oxidase (Stewart, 1974). Algal chloroplasts also contain plastocyanin, a blue copper protein found to mediate photosystem activity (Katoh, 1960).

Chemical speciation figures importantly in trace element requirements. Barber and Huntsman (1978) described copper ion activity as the "inherent toxic factor in natural deep ocean seawater." Spencer (1957) and Davey et al. (1973) reported that the addition of chelates reduced toxicity by copper towards phytoplankton. Sunda and Guillard (1976) ascribed this toxicity reduction to decreased free Cu^{2+} ion activity. Complexed copper is evidently not toxic to the diatom Thalassiosira pseudonana and the green alga Nannochloris atomus.

Similarly growth can be responsive to changes in copper concentration, because division rates for the unicellular Monochrysis lutheri inversely related to free Cu^{2+} concentrations (Sunda and Lewis, 1978).

Ranges of copper concentrations prescribed for different culturing formulations are relatively narrow. Toerien (1971) suggested 0.2 nM Cu_T while Jorgensen (1962) prescribed 60 nM copper. Natural levels in seawater range between 1 and 10 nM Cu_T. Such low concentrations may present control problems. Preparing nanomolar levels of a nutrient in a liter of medium requires microliter spiking volumes if a millimolar copper stock solution is used. Aerosol particle deposition may contaminate cultures with copper, especially in urban areas where industrial and automotive exhausts augment natural aerosol densities. North (1978b) found that a concentration of only 100 nM Cu_T inhibited growth among Macrocystis juveniles in enriched seawater.

Iron.

Allen (1914) recognized iron as an essential algal micronutrient. Iron atoms are structural components of heme (e.g. cytochromes) and non-heme (e.g. ferredoxin) molecules. Cytochromes mediate redox processes in both photosynthetic and respiratory systems. Ferredoxin is involved in the photoreduction of coenzyme NADP to NADPH (Powls et al., 1969). Chlorophyll synthesis and nitrogen assimilation have also been linked to iron metabolism (Rains, 1976).

Price (1968) found that cellular iron was primarily concentrated in chloroplasts. Iron deficiency in plants produces chlorosis, wherein green parts of the plant become

blanched. Iron deficiency in Macrocystis juveniles significantly reduced growth rates yielding "signs of unhealthiness" (North, 1977). Unlike copper, iron toxicity occurs rarely, if at all. This could arise from the variety of iron uses in plant metabolic systems or simply because Fe(III) is sparingly soluble in seawater.

Intraspecies variation in iron uptake efficiency was demonstrated by Kirkby and Mengel (1967) on tomatoes. Interspecies variations in iron uptake efficiency was proposed for planktonic algae by Chu (1942). Prescribed iron concentrations for algal culturing media ranged from 20 nM to 1.8×10^5 nM (Muller, 1962 and Manahan, 1973). This enormous range reflects our ignorance of iron requirements among algae.

Algal uptake is undoubtedly hindered by the strong tendency of Fe(III) to precipitate in seawater thus associating with the suspended particulates. Without organic chelates, iron exists in suspension primarily as amorphous $Fe(OH)_3$. Organic chelates (e.g. citrate and EDTA) solubilize iron precipitates in culture media. Although Harvey (1937) reported that diatoms assimilate colloidal or particulate ferric hydroxide, whether particulate iron can be assimilated by algae is still open to question (Manahan and Smith, 1973).

The availability of iron chelates to algae is not well understood. Iron bound to humic acids was not available to

the green alga Scenedesmus obliquus (Giesy, 1976). Iron requirements for Chlorella, however, were unaltered by the presence of the chelate EDTA. Romheld and Marschner (1979) claimed that plants were able to take up chelated as well as free ionic iron. Romheld used a double-labelled iron chelate (^{59}Fe-EDDHA-^{14}C) to show that: 1) plants split very stable iron chelates by first reducing the ferric chelate to the less stable ferrous chelate, and 2) the ability to break metal-chelate bonds increased under iron deficiency.

Manganese.

Since Hopkins' (1930) work on Chlorella, several investigators have determined that various phytoplankton require manganese. Electron transfer from water to pigment P680 (oxygen evolving photosystem II) required Mn^{2+} (Cheniae and Martin, 1969). Teicher-Zallen (1969) observed that manganese deficiency disrupted chloroplast membrane structure, inhibiting photosystem II but not photosystem I. Manganese is also involved in various enzyme systems (e.g., NAD-malic enzyme).

Mn_T in culture media formulations has ranged from 10 nM to 3.3×10^5 nM (Harvey, 1947 and Jorgensen, 1962). As little as 10-40 nM Mn_T produced vigorous growth of Chlamydomonas and Cryptomonas, (Harvey, 1947). 100 nM Mn_T was sufficient for autotrophic growth by Chlorella pyrenoidosa (Walker, 1953 and Eyster et al., 1958), while deficiency symptoms were

observed below 0.1 nM in heterotrophic cultures. Similar results were reported for C. vulgaris (Reisner and Thompson, 1956). Mn^{2+} were more important in light reactions than in dark reactions. Mn_T values ranged from 5nM to 22 nM for seawater analyzed by ultraclean techniques (Knauer, 1978). Mn_T levels above 20 nM were toxic to phytoplankton photosynthesis. Seasonal fluctuations in manganese concentration of water from 300 m deep correlated with fluctuations in growth rates of juvenile Macrocystis plants (North, 1978a). Terrestrial plants exposed to excess manganese in acidic soil exhibited chlorotic systems. Excess copper, nickel, zinc, as well as manganese, induced iron chlorosis (Brown et al., 1972). Mn deficiency in Chlorella raised the sensitivity of chlorophyll a to destruction by light, while increasing chlorophyll content (Eyster et al., 1958 and Alberts-Dietert, 1941). Chlorotic effects were not observed, but destruction of chlorophyll a under intense light could secondarily have produced chlorosis.

The optimal concentrations for manganese may be lower than for iron because: 1)Mg^{2+} may substitute for Mn^{2+} in many enzymatic reactions under manganese deficiency, and 2) Mn^{2+}-chelates generally have lower stability constants than Fe^{3+}-chelates.

Zinc.

The essential nature of zinc in algal nutrition was first demonstrated for Stichococcus bacillaris by Eiler

(1926). The primary biochemical role of zinc is in auxin metabolism. Tsui (1948) found that zinc was required for tryptophan synthesis (tryptophan is a precursor of auxin indolacetic acid, [IAA]). Zinc is also a cofactor in numerous enzyme systems (e.g. carbonic anhydrase) and has been shown to affect cytochrome-c synthesis.

About one micromolar zinc concentrations are needed for algal growth in synthetic media (Provasoli and Pintner, 1953). Concentrations below 100 nM produced zinc deficiencies in Chlorella pyrenoidosa (Walker, 1954). Zinc deficiency symptoms in phytoplankton include: 1) reduced cell division rate, 2) decreased chlorophyll formation (hence less photosynthetic activity), and 3) disappearance of cytoplasmic ribosomes (Prask and Ploche, 1971).

A few cases of zinc toxicity have been recorded for plants. For example, zinc toxicity reduced phosphorus and iron levels in plants (Adriano et al., 1971). Also, one micromolar zinc added either to 300 m or to surface seawater (background zinc concentration approximately 100 nM) reduced growth rates of Macrocystis juveniles (North, unpublished).

Concentration levels of approximately 1 μM total zinc (Zn_T) have been consistently prescribed for algal culture media. This is an order of magnitude greater than typical seawater values (Brewer, 1975), but may be necessary because

of the high levels of chelating agents typically added.

Trace Element Interactions.

Relationships between trace nutrients fall into two categories: 1) two or more micronutrients mediate a single process, or 2) micronutrient I actively stimulates or suppresses a process involving another trace substance, micronutrient II. The first case may produce a "limiting factor" condition when availability of one micronutrient is inadequate. The second case produces an interactive condition wherein the simultaneous addition of micronutrients I and II can yield greater or lesser results than the additive response from independent additions of I and II.

In an example of the first case, test plants became chlorotic in acidic soils rich in micronutrients (Somer and Shive, 1942): iron was inadequate in the soil relative to the other micronutrients. Chlorosis was eliminated by spraying the plants with iron solutions. The second case was demonstrated with oats grown in peat (Chesire et al., 1967). Copper and iron additions to the peat produced increased oat yields, but copper or iron additions alone had no effect. Scherfig et al., (1973) noted a similar effect with the green alga Selenastrum capricornutum. Increases in growth rate were noted in cultures when Mn_T was doubled, when Fe_T was doubled, and when Fe_T and Mn_T were simultaneously doubled. In the last case, however, the increased growth did not

exceed that which was observed when Fe_T alone was doubled (Figure 2.1) Speciation for these media were calculated (Table 2.1). Iron addition affected free ion concentration of manganese and vice versa. When Fe_T and Mn_T were doubled simultaneously 25-fold and 24-fold increases occurred in iron and manganese free ion concentrations respectively. Stimulatory effects of increased free iron concentrations were evidently offset by an inhibitory increase in free manganese concentration. Examples of manganese-induced chlorosis have also been reported for terrestrial plants (Somer and Shive, 1942, Hanger, 1965).

Nitrate.

Amino acid and nucleic acid synthesis impose demands for nitrogen. Biologically available nitrogen in seawater exists primarily as nitrate and ammonium ions with lesser amounts of the nitrite ion. Nitrate assimilation by algae involves the reduction of nitrate to ammonium, and this requires energy. Some species exploit low levels of reduced nitrogen first by mechanisms related to the reduction process which itself regulates nitrate uptake (Steward, 1974). Thus ammonium-N is preferentially used over nitrate-N by many microscopic and macroscopic algae (Ludwig, 1938, and Haines and Wheeler, 1978). Macrocystis is, however, one of several species that shows no preferential uptake of ammonium over nitrate (Haines and Wheeler, 1978). In fact, high concentrations (e.g., 30 μM) of ammonium have retarded

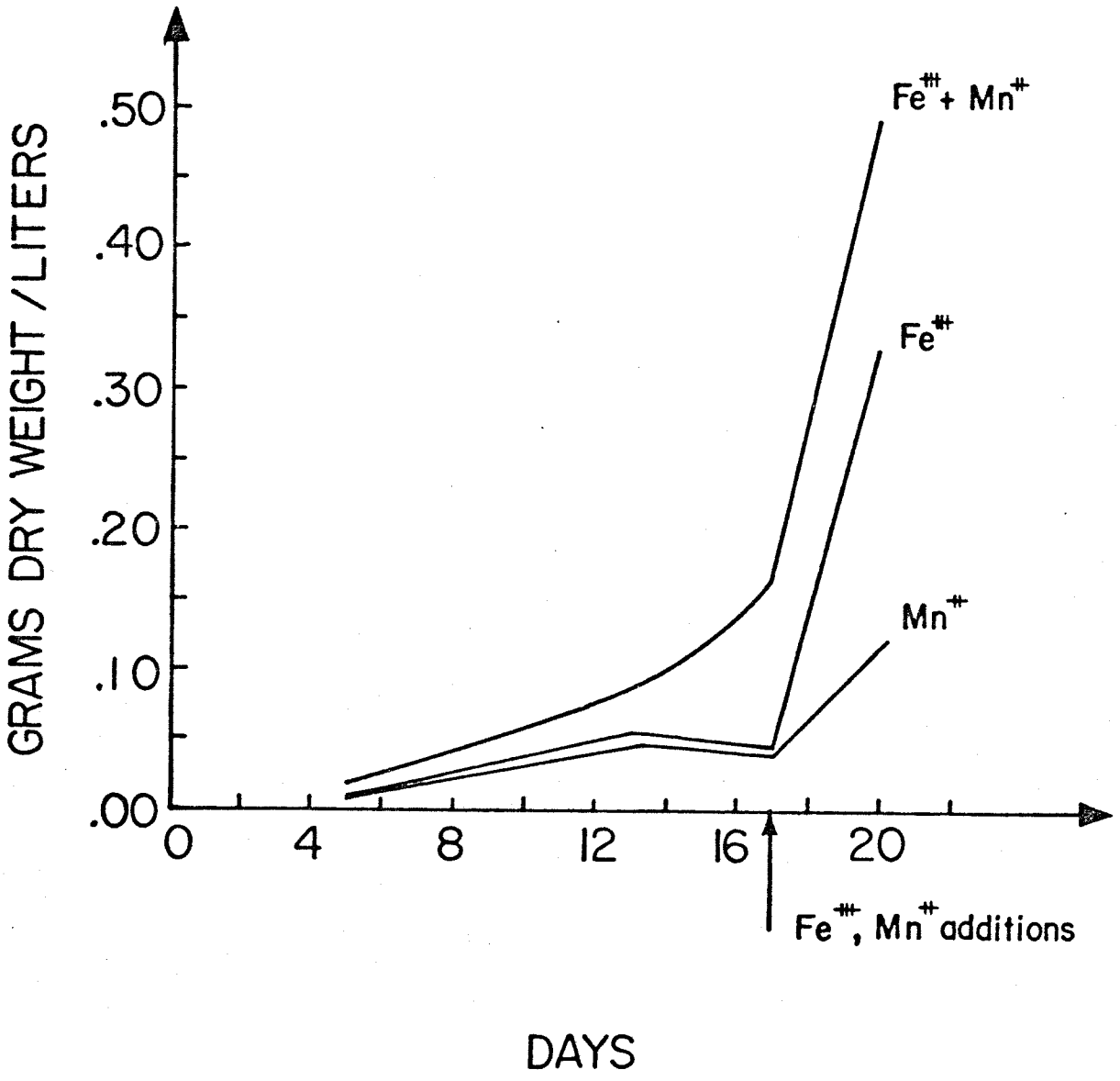


FIGURE 2.1. EFFECT OF IRON AND MANGANESE ADDITION TO UNMODIFIED PAAP MEDIUM (Scherfig et al., 1973).

Table 2.1. SPECIATION OF SOME TRACE METALS AND LIGANDS IN THE PAAP MEDIUM *
(Effects of Iron and Manganese Additions)

	Initial Conc. (M)	Computed Free Ion Activity (M) No Add.	Fe Add.	Mn Add.	Fe+Mn Add.	Major Species Initial Medium		PAAP+Fe+Mn	
Iron	2×10^{-6}	3.5×10^{-21}	9.0×10^{-21}	1.5×10^{-20}	8.7×10^{-20}	FeEDTA	100%	FeEDTA	100%
Manganese	7×10^{-6}	8.9×10^{-9}	1.2×10^{-8}	7.4×10^{-8}	2.1×10^{-7}	MnEDTA	100%	MnEDTA $MnCl^+$	95% 3%
Cobalt	2×10^{-8}	1.6×10^{-13}	2.1×10^{-13}	6.7×10^{-13}	2.0×10^{-12}	CoEDTA	100%	CoEDTA	100%
Copper	2.2×10^{-10}	5.5×10^{-18}	7.1×10^{-18}	2.3×10^{-17}	6.9×10^{-17}	CuEDTA	100%	CuEDTA	100%
Zinc	8×10^{-7}	3.2×10^{-12}	4.1×10^{-12}	1.3×10^{-11}	4.0×10^{-11}	ZnEDTA	100%	ZnEDTA	100%
Molybdate	1×10^{-7}	1×10^{-7}	1×10^{-7}	1×10^{-7}	1×10^{-7}	Free	100%	Free	100%
Arsenate	1×10^{-8}	1.2×10^{-11}	1.2×10^{-11}	1.2×10^{-11}	1.2×10^{-11}	$HAsO_4^-$	100%	$HAsO_4^-$	100%
EDTA	2×10^{-5}	9.3×10^{-12}	7.2×10^{-12}	2.2×10^{-12}	7.4×10^{-13}	CaEDTA MnEDTA	47% 37%	MnEDTA FeEDTA	71% 21%
Nitrate	1×10^{-3}	1×10^{-3}	1×10^{-3}	1×10^{-3}	1×10^{-3}	Free	100%	Free	100%
Selenite	1×10^{-8}	5.4×10^{-9}	5.4×10^{-9}	5.4×10^{-9}	5.4×10^{-9}	Free $HSeO_3^-$	54% 46%	Free $HSeO_3^-$	54% 46%

pH = 8.0

I = 0.7 M

$-\log (P_{CO_2}) = 3.5$

*Analytical concentrations from Scherfig (1972).

growth in Macrocystis juveniles (North, 1979). Seawater nitrate concentrations vary seasonally in coastal kelp beds off southern California (Jackson, 1977). Lows occurred during the summer and highs during spring upwelling (>1 μM [NO_3]). Nitrate concentrations of 40 μM are common in deep seawater (Brewer, 1975).

Nitrogen as $NaNO_3$ or KNO_3 is usually added to synthetic seawater at extremely high concentrations. Provasoli (1957) specified 5900 μM in his ASP-2 medium. Manahan and Smith (1973) used N_T level of 10^4 μM while studying algal copper requirements. Such enormous concentrations of nitrate are probably not detrimental to species that possess the nitrate reduction mechanism for uptake regulation. North (unpublished) has noted some short duration inhibition when juvenile Macrocystis (a plant lacking the regulatory mechanism) is first introduced to 30 μM nitrate.

Temperature.

Equilibrium constants K and reaction kinetics are temperature dependent. Rates usually increase with temperature for enzymatic reactions below 40°C, (Buetow, 1962 and Parekh et al., 1969). Temperature shock can inhibit photosynthesis by Chlorella (Pirson et al., 1959).

Studies of temperature requirements, tolerance and adaptibility indicate that optimal ranges can vary within and between species. Thus optimal temperatures of 25, 30,

and 39°C were determined for different strains of Chlorella pyrenoidosa (Sorokin, 1959 and Lorenzen, 1963) and 7°C for the marine diatom Fragilaria sublinearis (Bunt, 1965).

Optimal temperature for development of various Laminarian gametophytes depended upon the seawater temperature at the sporophyll collection site (Lüning and Neushul, 1978). Optimal temperatures for gametophytes from central California were 12°C, compared to 17°C for gametophytes from southern California.

In this thesis, temperature was selected as a culturing variable to: 1) compare relative sensitivities of Macrocystis gametophytes to temperature versus micronutrient levels, and 2) determine the optimal temperature range for southern California Macrocystis gametophytes.

CHAPTER 3

A CHEMICALLY DEFINED CULTURING MEDIUM

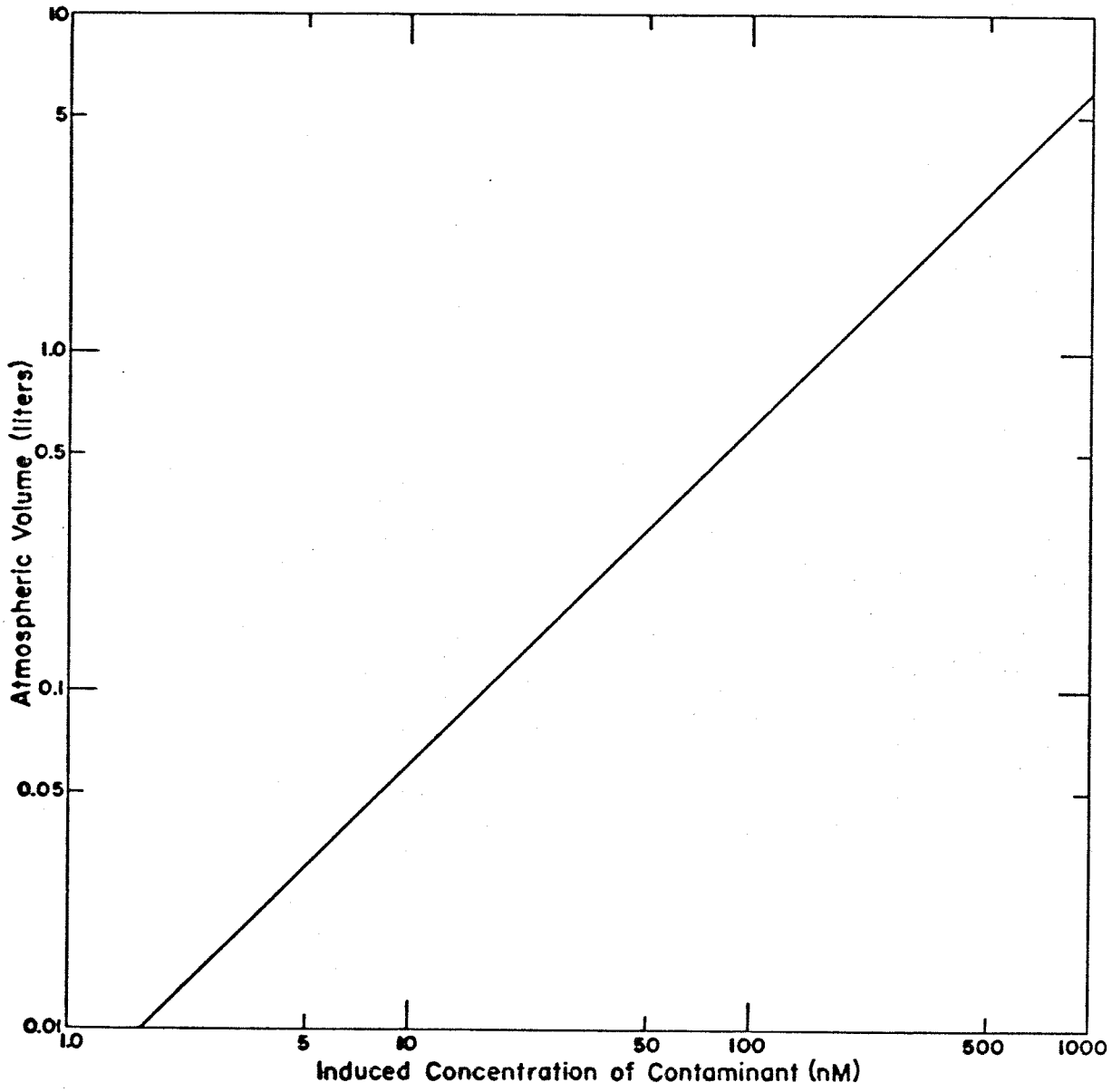
Introduction.

The control level for any variable v is comprised of three elements: $X_v = C + P + R$. C is predetermined. We refer to C as a variable "setting." P, known as the pseudo-random part (Lucas, 1964), is generated by experimental design which seeks to reduce pseudo-random variation. R is the truly random element, representing an inherent limit to control of X_v. Low micronutrient concentrations exacerbate the relative magnitude of the pseudo-random component. Attempts to minimize P effects on micronutrient control levels are described below. Beyond the scope of this thesis, R effects are dealt with by statistical thermodynamics.

Selection of Culture Medium.

Reports in the literature have described occasional success in plant culturing in aqueous media, but these results have often been difficult to evaluate, apply, or even reproduce because of uncontrolled and/or unknown background levels of trace metals (Morel et al., 1979). Poorly defined background concentrations of trace metals and of organic matter have also complicated the calculation of chemical speciation both in seawater and in nearly all culturing media. For example, data obtained by Hidy (1974) (Figure 3.1) indicate that less than one liter of urban air contains

Figure 3.1. Volume of urban atmosphere containing sufficient aerosol particles less than one micron in diameter to produce a zinc contamination of the indicated concentration in 30 mL of medium.



sufficient submicron aerosol particles (most of which are beyond light microscope resolution) to produce zinc contamination in a Petri dish culture equivalent to the natural background levels of this element in seawater (Brewer, 1975). Morel et al. (1979) cited an example where a synthetic medium was contaminated by direct addition of reagent grade chemicals. It is therefore essential to apply ultraclean techniques to guarantee integrity of defined growth media. Work by Anderson et al. (1978) represents a study involving precise chemical definition of algal growth media.

This thesis describes ultraclean techniques we have developed for preparing media and operating the culture system. The basal medium selected was Aquil, an artificial seawater first formulated by Morel et al., (1975). Twenty-three salts, primarily metal-chlorides and sodium-ligands, were used in Aquil preparation (Table 3.1). The basic Aquil formulation specifies major inorganic salt concentrations which correspond closely to principal composition of seawater (EPA, 1971). For this work, the original production system by Morel et al. (1975) was modified to permit variable macronutrient and trace element concentrations (Appendix I). All chemicals were reagent grade or better. Solutions of major salts and macronutrients were passed through columns of Chelex-100 (Bio·Rad Laboratories, Richmond, CA, 100-200 mesh) to remove cationic trace element impurities (see Figure A, Appendix I).

Table 3.1. Aquil Salts

I. Major salts (2xS.O.W.): Initial stock volume = 25 liters. Dilution factor = 2.

Mass, g	Salt	Gram formula weight, g	Final molarity	-log M
1226.5	NaCl	58.44	0.42	0.38
77.0	$CaCl_2 \cdot 2H_2O$	147.02	1.1×10^{-2}	1.98
5.0	KBr	119.01	8.4×10^{-4}	3.08
0.15	NaF	41.99	7.1×10^{-5}	4.15
35.0	KCl	74.56	9.4×10^{-3}	2.03
1.5	H_3BO_3	61.83	4.9×10^{-4}	3.31
204.5	Na_2SO_4	142.04	2.9x10-2	1.54
10.0	$NaHCO_3$	84.01	2.4×10^{-3}	2.62
0.85	$SrCl_2 \cdot 6H_2O$	266.62	6.4×10^{-5}	4.20
555.0	$MgCl_2 \cdot 6H_2O$	203.31	5.5×10^{-2}	1.26

II. Macronutrient additions (variable)

A. K_2HPO_4

B. $NaNO_3$ or NH_4Cl.

III. Trace element additions (variable)

A. Metals - Co(II), Cu(II), Fe(III), Mn(II), Ni(II), Zn(II) (added as chloride salts)

B. Ligands - I^-, MoO_4^{2-}, SeO_3^{2-}, AsO_4^{3-}, $EDTA^{2-}$ (added as sodium salts.)

Chemical Equilibrium Program.

At thermodynamic equilibrium, chemical speciation of Aquil components is completely determined. Computer programs such as REDEQL2 (McDuff and Morel, 1973) can be used to calculate this speciation. Conventional analytical methods for determining trace element speciation in chemically complex media are either unsatisfactory or nonexistent.
Validity of the REDEQL2 approach depends upon: 1) accuracy of analytical concentrations during media preparation; 2) accuracy of the required thermodynamic data (i.e., equilibrium constants); and 3) kinetics of reactions among Aquil components (i.e. whether thermodynamic equilibrium is achieved).

Precautions taken to minimize errors in media preparation were discussed above. Equilibrium constants and solid formation constants in REDEQL2 were corrected for ionic strength by the Davies Equation (McDuff and Morel, 1973). Equilibrium constants used in the REDEQL2 program were typically determined at room temperature (25°C). Culturing experiments described in this thesis, however, were conducted at 10-18°C. A new subroutine, TEMCOR, was therefore incorporated into REDEQL2 to examine temperature effects on chemical speciation (Appendix II).

Iodine is present in seawater as iodide, I^-, and iodate, IO_3^- (Brewer, 1975). The iodide-iodate redox couple was not employed in REDEQL2 computations. I_T was fixed at 100 n$\underline{M}$ in all Aquil culturing experiments. The Mn(II)-Mn(IV) redox

couple was also not employed because the oxidation of Mn(II) to Mn(IV) is evidently slow relative to gametophyte culturing periods (Morgan, 1967, Matsui, 1976).

By employing an equilibrium program like REDEQL2 to compute chemical speciation for Aquil experiments, there was an implicit assumption that metal uptake by Macrocystis gametophytes did not significantly disturb any chemical equilibria (i.e., ion association reactions involving micronutrients in Aquil are fast relative to metal uptake by Macrocystis.) Metal uptake rates estimated for gametophyte batch cultures (see Appendix IV) suggest that this assumption was valid. That is, estimated dissociaticns rates for the metal-EDTA complexes in Aquil were orders of magnitude faster than computed rates of uptake by gametophytes.

Ultraclean Technique.

Rigorous attention to cleanliness during all phases of experimentation was absolutely essential to success of this study. Distilled water of 18 megohm resistance (called Q water, equivalent to triple-distilled in quartz stills), prepared by Millipore RO-RQ systems, was always used in preparing stock solutions, media, and during the cleaning and rinsing of labware. All glassware and plasticware were acid washed as prescribed in Appendix I. Media were stored in Teflon or polyethylene bottles enclosed in plastic wrap (Subramaniam et al., 1978, and Moody and Lindstrom, 1977). Storage times never exceeded 14 days. All preparative operations were conducted in

a clean room equipped with a laminar flow hood and with incoming air filtered to remove 99.9% of particles greater than 0.3 microns in diameter (Environmental Air Control, Inc., Hagerstown, MD, Model FTC75SL). All exposed surfaces in preparative and culturing areas which were not plastic were either painted with white epoxy, or latex paint, or lined with polyethylene sheeting. Painted and lined surfaces were routinely swabbed with methanol, then redistilled 0.1 N HCL, then Q water, every seven days. Polyethylene gloves, laboratory coats, and special shoes were worn during all clean room work. No metallic apparatus or instruments were even allowed to contact the solutions or media. Media were occasionally checked for purity by atomic absorption spectrophotometry for copper (<1n$\underline{M}$) before trace elements were added to the mixtures (Table 3.2)

Experimental apparatus which was not commercially available was fabricated at the Keck Engineering Laboratory Shop. For example, to avoid possible contamination during transfers of liquids, a filtering unit was devised to allow millipore filtration of samples directly into Teflon storage bottles of variable size instead of first into the conventional Erlenmeyer vacuum flask (Figure B, Appendix I). This unit was also used for seawater and for final Aquil sterilization. Apparatus were machined, insofar as possible, from TFE-Teflon rod stock, polyethylene and Lucite. Plans for fabricated parts not shown in this thesis are available from the author.

CHAPTER 4

GAMETOPHYTIC CULTURING APPARATUS

Introduction.

Procedures for culturing Macrocystis gametophytes have been described by Anderson and North (1966), Lüning and Neushul (1978), and Devinny and Volse (1978). Many experimental procedures were common to these studies:

1. sporophylls (specialized reproductive blades) were collected from adult plants;
2. blades were wiped or blotted to remove bacterial-infested mucous;
3. fertile blade portions, the sori, released biflagellated spores after immersion in culture media at 8 to 10°C for approximately 30 minutes;
4. Petri dishes containing microscope slides (or cover slips) were innoculated with released spores that then settled, adhered, and underwent gametophytic development; and
5. sample slides or slips were periodically examined and preserved.

These general procedures were also followed during the present study and any significant deviations from these procedures will be described in the appropriate section below.

Initial Culturing System.

Processes controlling metal speciation in natural waters include: 1) inorganic complexation; 2) organic complexation: 3) precipitation; and 4) adsorption. REDEQL2 computations were used to determine the magnitude of the effects of the first two processes. Presence of precipitates was avoided by: 1) controlling metal ion concentrations with the chelate EDTA; 2) sterilizing culture media by filtering vs. autoclaving, and 3) routine pH measurement and adjustment as necessary during experiments. Teflon, polyethylene and high purity quartz were used wherever possible to minimize metal adsorption.

Initial experiments for this thesis were conducted at Hopkins Marine Station in Pacific Grove, California during the summer of 1977. A 2' x 1½' x 2' cabinet contained the entire culturing system (Figures 4.1 and 4.2). Portability was an important design criterion. A peristaltic pump (Buchler Instruments, Fort Lee, NJ., Model 2-6100) circulated the temperature controlled medium down a Teflon trough, thence by gravity flow to a Teflon reservoir. Two 15-watt Cool-White fluorescent bulbs provided light.

The apparatus was placed in a coldroom or refrigerator so that ambient temperatures were below control temperatures. Thus, temperature regulation involved only heating. The sensing element (B. Braun, San Francisco, CA, Model 16-100A)

Figure 4.1. Initial culturing system used at Hopkins Marine Station and Kerckhoff Marine Laboratory.

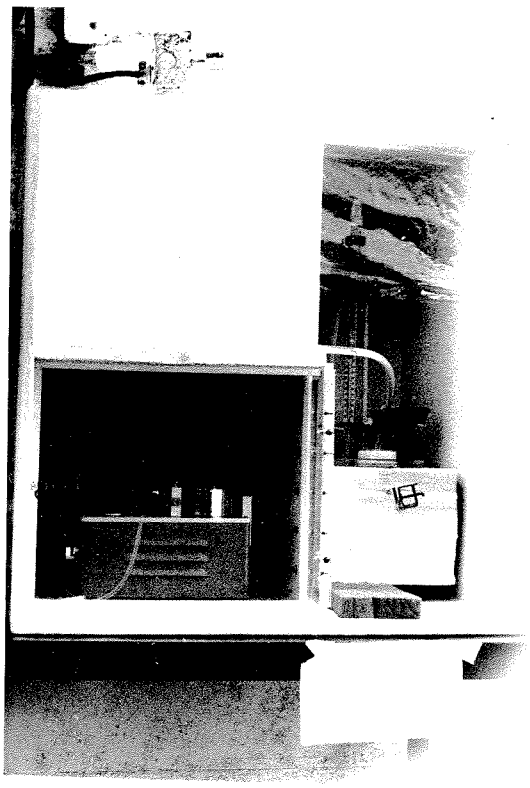
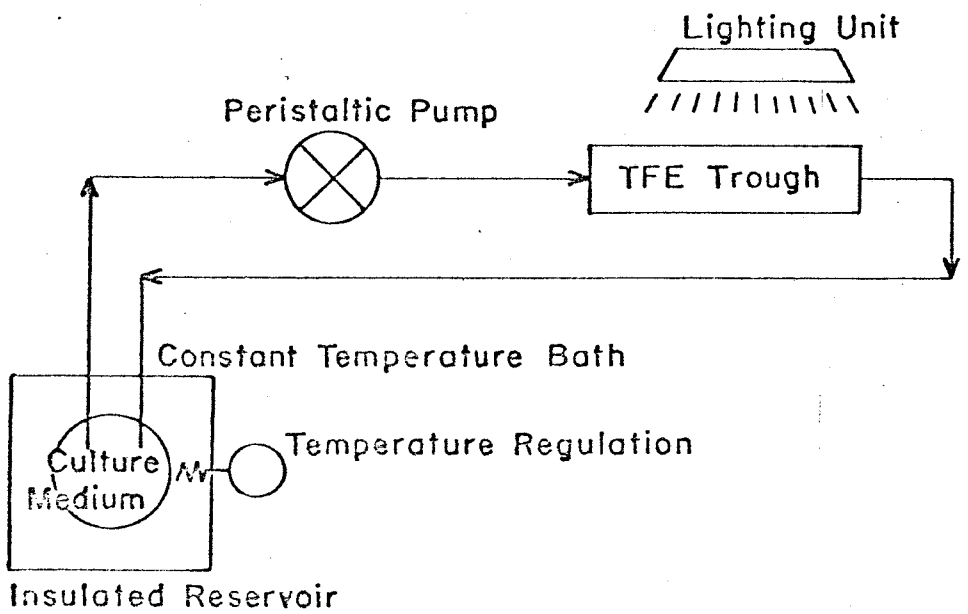


Figure 4.2. Schematic diagram of the inital culturing system.



was used with a mercury plunger power relay (PSG Industries, Inc., Perkasie, PA, Model PRSS) and a 250-watt Vycor immersion heater (Corning Glass Works, Corning NY, Model 16790). The culturing trough was machined from 1¼" TFE-Teflon sheeting (Figure 4.3). Cover slips (18x18 mm) were secured in the trough by two 1/32" slots, cut by a Woodruff keycutter.

An illuminated spore suspension was poured into the trough under still conditions. Spores were allowed 30 hours to settle and attach to slips. A cover slip was removed each day to inspect development of attached gametophytes.

Pumping was then initiated at a rate of 50 mL/min. Average flow velocity through the trough was 1 cm/sec although the reported minimum velocity required to achieve maximal nitrate uptake in adult Macrocystis sporophytes is 4 cm/sec. (Wheeler, 1978). Attached spores were scoured from cover slips when average velocities exceeded 1 cm/sec., even after a 48-hour settling period. It appeared possible that this low circulation rate might produce a diffusion-limited condition. Computed theoretical diffusion rates were compared with uptake rates estimated from observed growth rates and total cell composition of juvenile Macrocystis sporophytes (North, 1977). The calculated theoretical diffusion rates were consistently lower than estimated uptake rates (Appendix III). A portion of the uptake is represented by ions adsorbed and concentrated in cell walls by

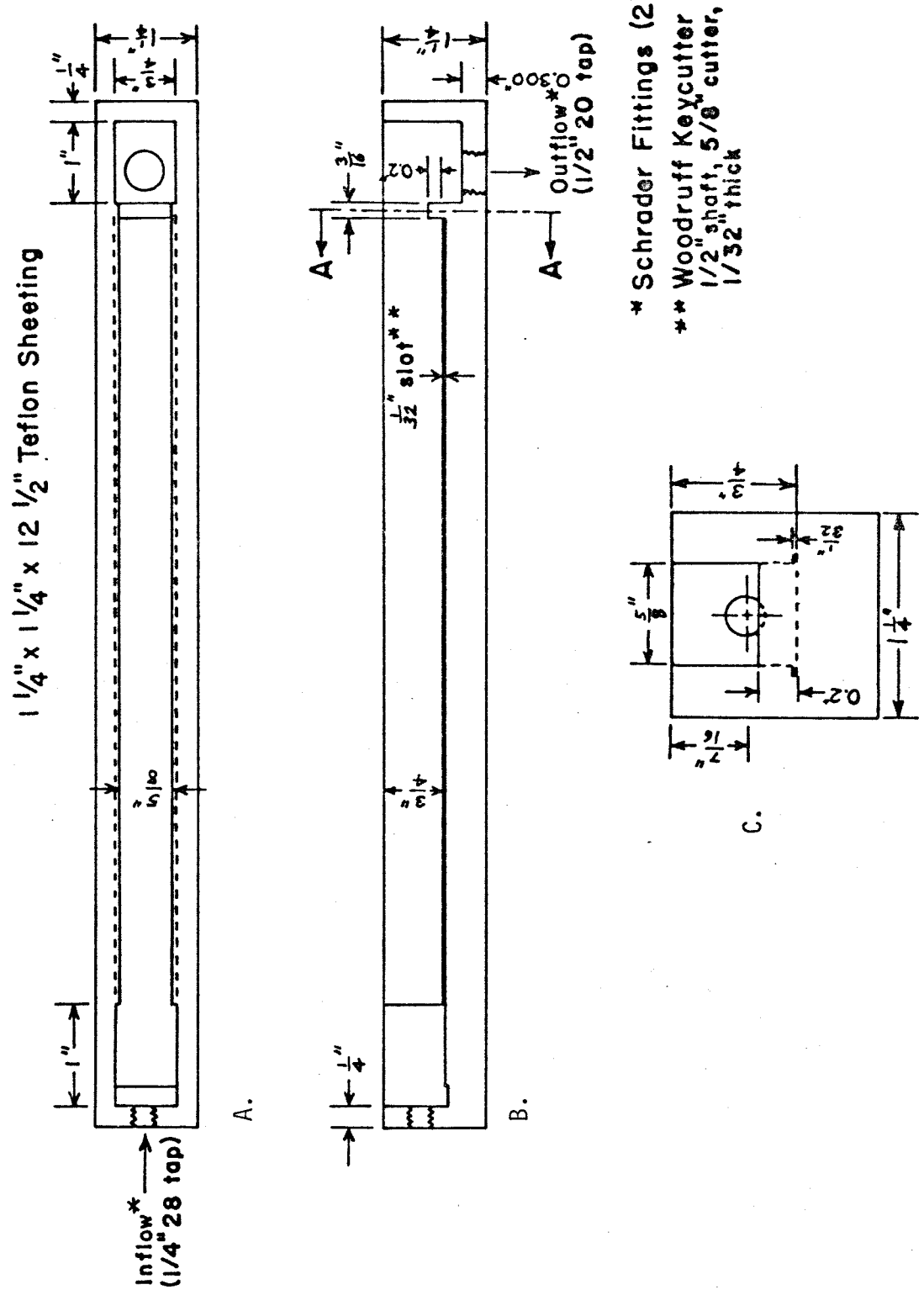


Figure 4.3. Diagrammatic details of the Teflon trough that held the cover slips supporting cultures. A. Plan view. B. Lateral cross section. C. End cross section.

polysaccharides such as alginic acid (Wasserman, 1948). Membrane permeability and active transport processes regulate uptake to the cell interior.

Dye experiments revealed parabolic velocity profiles laterally across the Teflon trough. If a diffusion-limiting condition had existed, developmental rates of gametophytes would have differed between the channel's center and either side. Cultures developed uniformly. Embryonic sporophytes appeared abundantly on the tenth culturing day throughout the culturing surface. A diffusion-limited condition did not appear to be present and use of the trough design was continued.

Modified Culturing System.

The trough system was transferred to a basement office near Kerckhoff Marine Laboratory after the summer at Hopkins Marine Station. Temperature control of the system was maintained by cold water bath which in turn was cooled by pumping refrigerated Q water through glass coils (13 mm O.D.) immersed in the styrofoam-insulated bath (Figure 4.4).

Continuous 24-hour illumination (approximately 14,000 ergs/cm^2/sec) raised the trough compartment air temperature to 30°C. Medium entered the trough at 12.5°C but left at 15.5°C. Vigorous bacterial growth near the trough drain apparently inhibited gametophytic development. Refrigeration was required to eliminate this temperature gradient. The system instead was modified (Figure 4.5).

Figure 4.4. Trough culturing system used at Kerckhoff Marine Laboratory but abandoned because undesirable temperature gradients were produced in the trough. Dashed lines denote separation of acid-cleaned culturing area from external laboratory space.

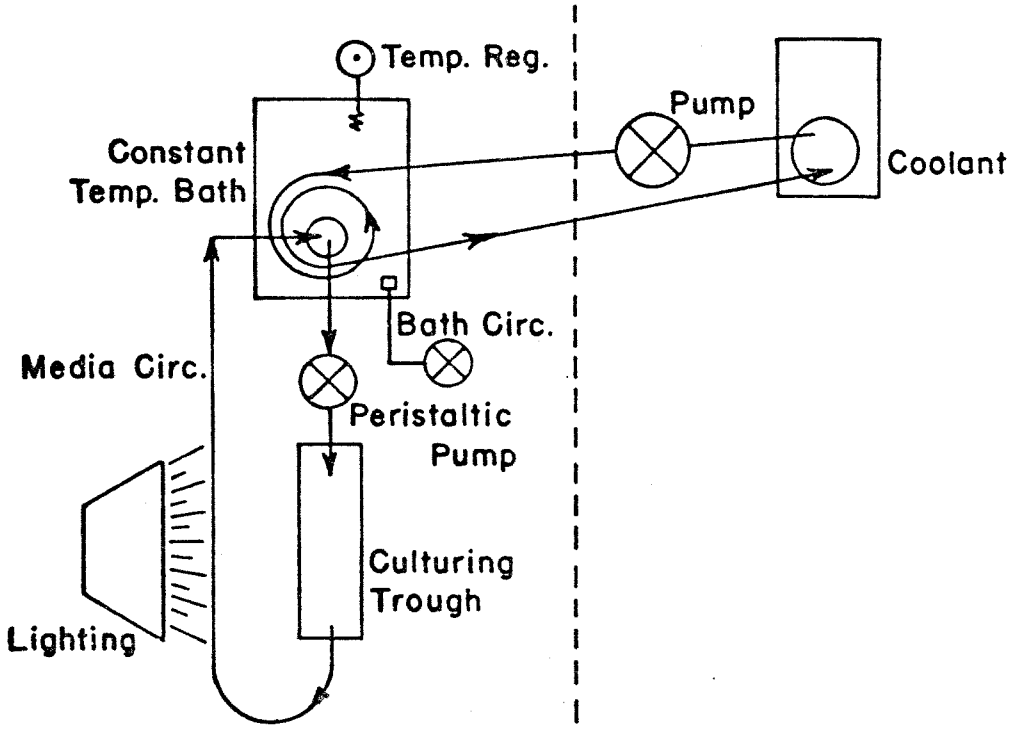
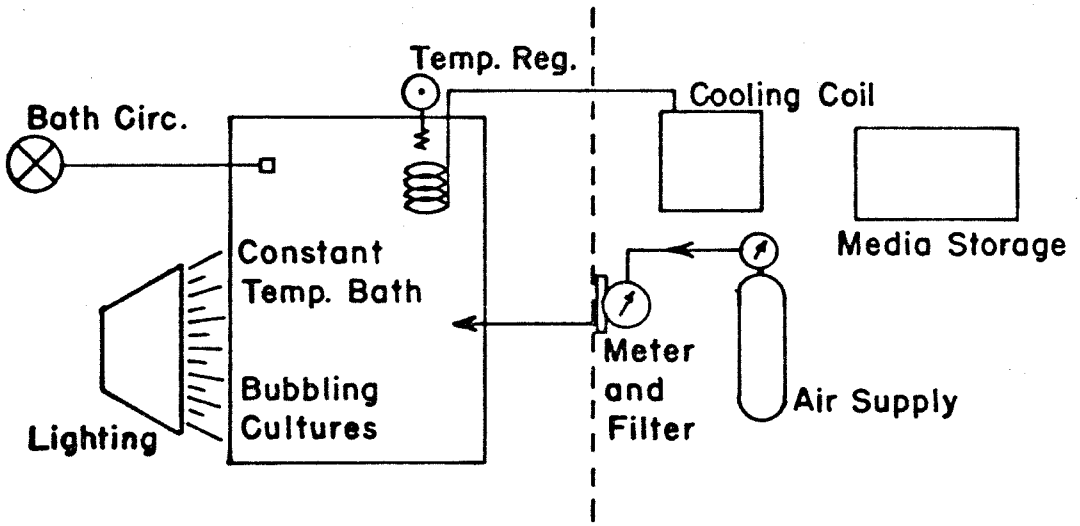


Figure 4.5. Culturing system used at Kerckhoff Marine Laboratory for the bulk of the culturing studies. Dashed lines denote separation of acid-cleaned culturing area from external laboratory space.

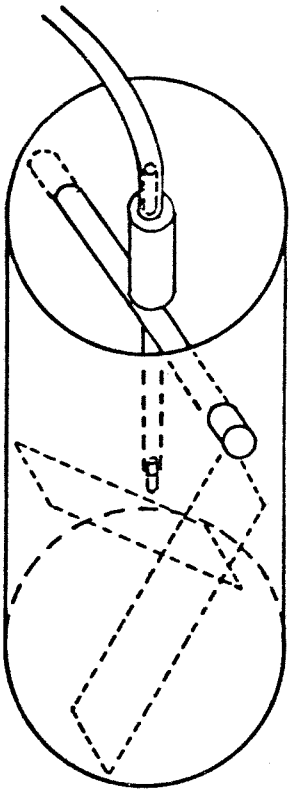


Troughs were replaced by 400 mL linear polyethylene (LPE) culturing vessels, each holding two microslide cultures (Figure 4.6). A Lucite rack holding eight of the 400 mL vessels (9 after Series I) was submerged in a temperature-controlled Q water bath (Figure 4.7). Teflon aerators maintained temperature uniformity both within vessels and within the Q water bath. The modified system eliminated: 1) the need for recirculating the medium, and 2) the temperature gradient within the culturing enclosure. The modified system also reduced the number of system components, space requirements, and maintenance costs.

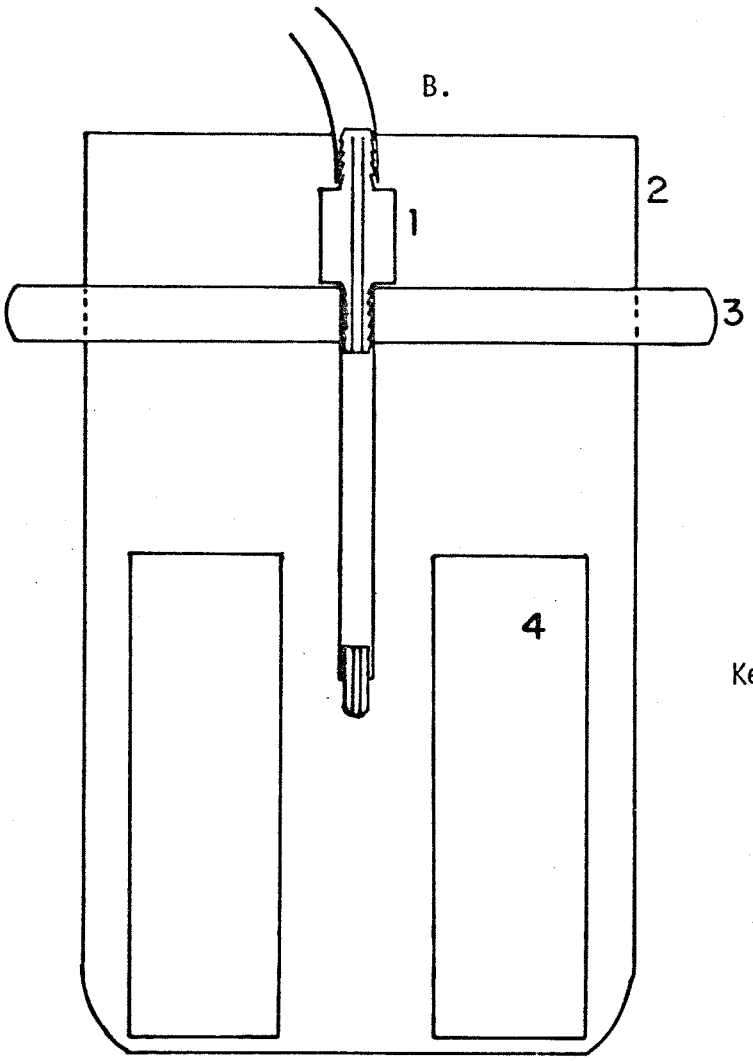
Culturing System Operation.

Macrocystis spores were released in Aquil ($10^{o}C$) from stirred, blotted, fertile reproductive blades (sporophylls) (Figure 4.8). Resulting spore suspensions were then stored in darkness for four hours in polyethylene graduated cylinders, permitting diatoms and debris to settle while the swimming biflagellated spores tended to remain suspended. Spore settling was induced by transferring suspensions to illuminated vessels containing microslides. Spores settled and adhered to microslides after 30 hours. Slides were then transferred to culture vessels containing 250 mL of medium, stirred by filtered air, supplied at 125 mL/min through specially fabricated Teflon aerators. Media were renewed every three days by suction through Teflon siphons to avoid vessel removal and to minimize microslide handling. Teflon

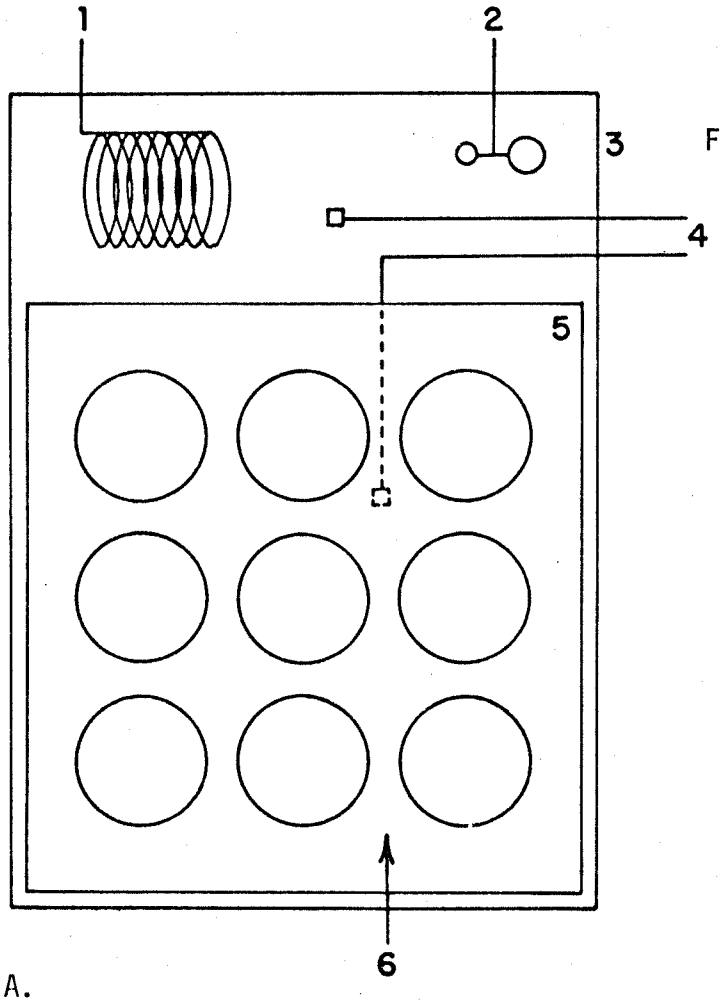
Figure 4.6. Gametophyte culturing vessel as used in the modified culturing system. A. Oblique view. B. Cutaway view (1:1 scale).



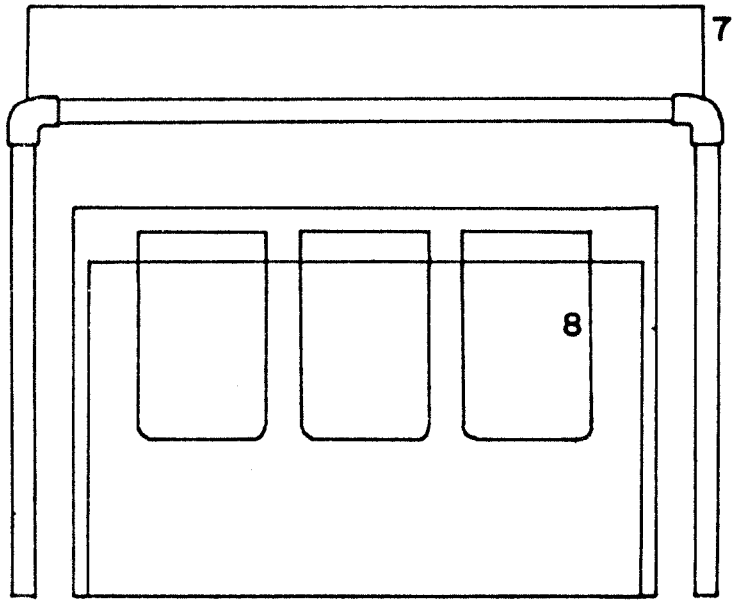
A.



Key:
1. Teflon aerator
2. Polyethylene culturing vessel
3. Teflon support rod
4. Microslide cultures.



A.

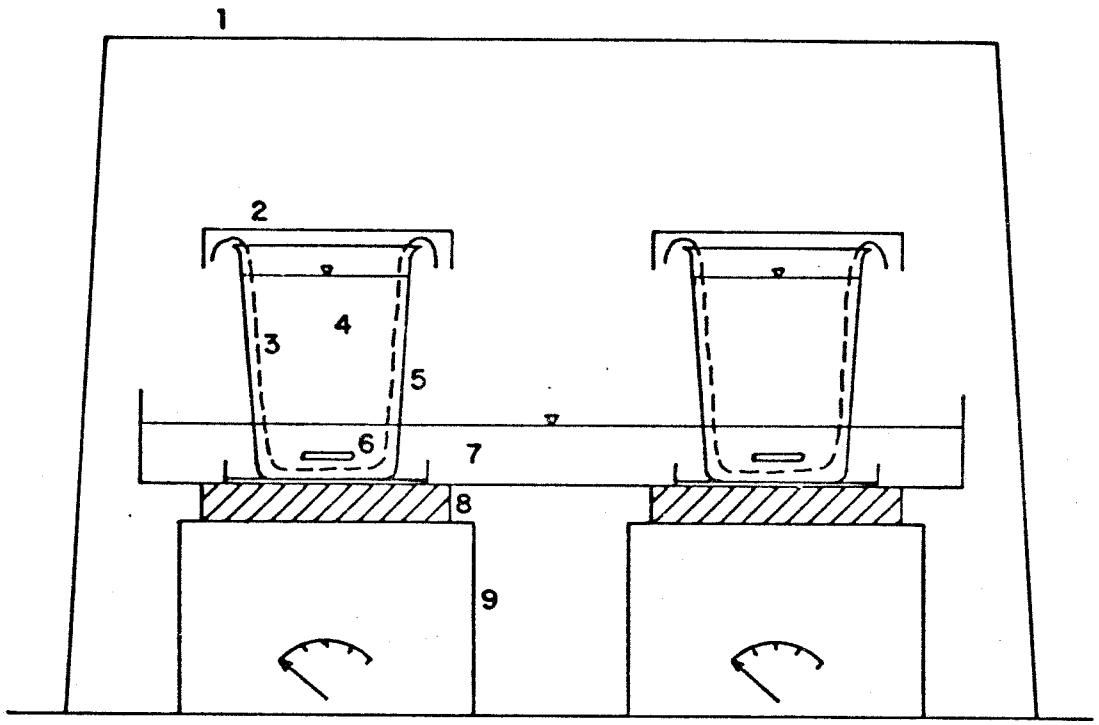


B.

Figure 4.7. Temperature controlled bath containing nine culturing vessels. A. Plan view beneath lighting unit not shown. B. Side view (1:4 Scale).

Key:
1. Cooling coil
2. Temperature regulation
3. Constant temperature bath
4. Bath circulation
5. Vessel-support stand
6. Metered aeration
7. Lighting unit
8. Culturing vessels.

Figure 4.8. Spore release system. Spore release operation was conducted in complete darkness.



Key:
1. Styrofoam cover
2. Plastic lid
3. Sporophylls
4. Spore suspension
5. Teflon beaker
6. Magnetic stirring bar
7. Cooling bath, Q water
8. Insulation pad
9. Magnetic stirrer.

forceps (J & H Berg Inc., South Plainfield, N.Y. Model W251056) and fabricated TFE spatulas were used when handling cultures.

Gametophytic development was followed for two weeks following spore release. Occurrences of gametogenesis and sporophyte production were noted. Gametophytic length also served as a measure of growth performance. Longest dimensions were determined by eyepiece micrometer for ten plants per microslide, selected by a technique based on a computer-generated series of random numbers. Significances of differences between means were analyzed by Student's t-test. Growth rate (change of length with time) tended to decrease during gametogenesis, affecting comparisons of length between cultures that matured at different times. In such cases, lengths were measured at the first indications of gametogenesis. In the absence of gametogenesis, gametophytic lengths were measured after two weeks.

Optimization method.

The Method of Steepest Ascent (Finney, 1960) was used to determine an optimal range for each experimental parameter. A starting point P_o, selected in n-dimensional treatment space, was presumed to produce a response Y close to the maximum response. Selection of P_o was based on information from previous nutrient studies. Let x_i = level of parameter i. Observations on Y, made at and near P_o, were used to calculate

the gradient $\partial \vec{Y}/\partial x_i$ at P_o. Point P_1 was chosen along the gradient at a suffieicnt distance from P_o to produce significant change in Y ($p < 0.05$). Observations on Y at and near P_1 determined the gradient $\partial Y/\partial x_i|_{P_1}$. The sequence was repeated until the gradient reversed direction.

CHAPTER 5

OPTIMIZATION EXPERIMENTS

Preliminary Experiments.

The initial culturing system (see Figure 4.1) was transported to Hopkins Marine Station in Pacific Grove, California where marine algae were studied during a 5-week summer course in 1977. Sporophylls were collected off Otter Point in Monterey Bay. Millipore-filtered (0.45μ), unenriched Monterey Bay surface water (5 m depth) served as the culturing medium.

Embryonic sporophytes appeared 11 culturing days after spore release (Figure 5.1). Similar development required approximately two months in experiments at Livermore Radiation Laboratories (Smith, personal communication).

Monterey Bay surface water samples were analyzed at Kerckhoff Marine Laboratory (KML) for NO_2+NO_3-N, NH_4-N and PO_4-P (Table 5.1) according to Strickland and Parsons (1972). Concentrations were below levels used in enriched seawater media during previous nutrient experiments at KML. A second sample of Monterey Bay surface water was collected for trace element analysis (Table 5.1). With the exception of copper, results showed consistently high micronutrient levels relative to other seawater samples. A test batch of gametophytes was cultured in a 1:1 mixture of surface and deep (300 m) water obtained 6 miles south of Newport Bay to compare to our results from Monterey. Development

Figure 5.1 . Photomicrograph of Hopkins Marine Station culture, two weeks after spore release (illustrated early embryonic development).

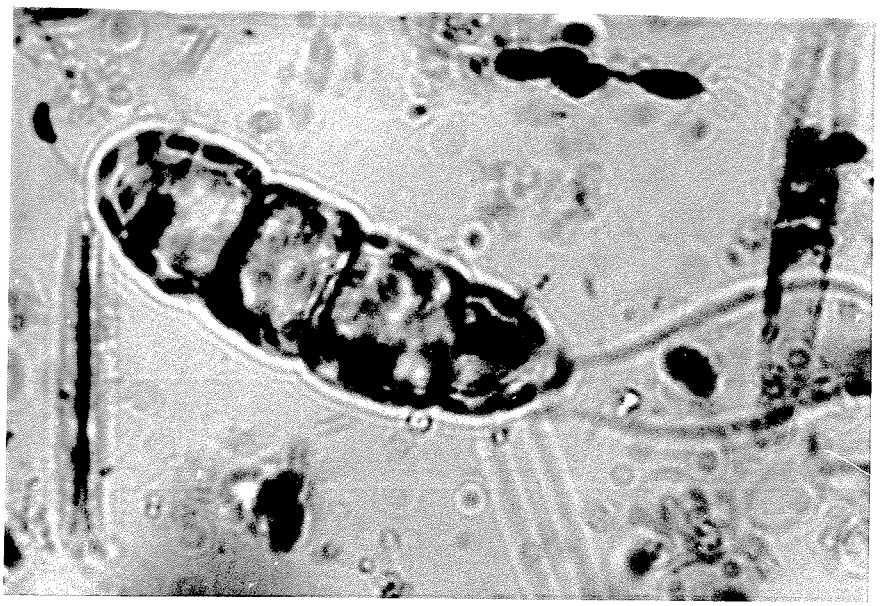


Figure 5.2 . Kerckhoff Marine Laboratory culture, two weeks after spore release. Development was scarcely beyond spore germination. Note reduction of diatom and fungal contamination due to differential spore suspension settling.

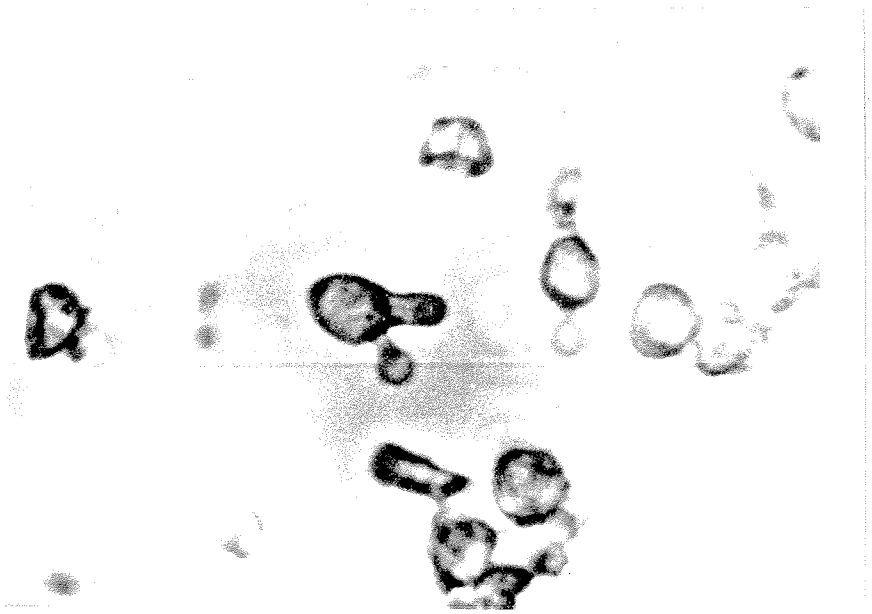


Table 5.1. Concentrations of several trace nutrients in various seawater samples used in laboratory Macrocystis culturing studies. All trace metal analyses were conducted by atomic absorption spectrophotometry (North, 1977).

Source of sample	Depth of sample (m)	Date	ug-at/L (Concentrations)			ng-at/L				
			NH_4	NO_3+NO_2	PO_4	Cu	Mn	Zn	Fe	Co
Crystal Cove Grid	45	7/15/76	0.34	11.70	1.20	2.3	1.9	58	22	
St. Croix	870	8/15/76	0.02	28.75	1.99	315.0*	2.7	22	54	
Triangle Station	300	9/18/76	0.30	24.46	2.36	23.0	0.17	26		1.7
Triangle Station	Ø	11/2-3/76	0.36	0.48	0.20	38.0	2.87	41	13	1.2
Pt. Loma	Ø	2/9/77	-	-	-	130.0	35	23	25	5.1
Triangle Station	300	5/5/77				11.0	7.3	15.3	22.4	11.0
Monterey Bay	5	8/23/77	1.1	6.24	1.09	15.7	22	61	72	30

*Unusually high value; may represent contamination

was slow during the 14 culturing days (Figure 5.2), so we decided henceforth to use Aquil.

The first experimental group determined optimal ranges for selected parameters: Co_T, Cu_T, Fe_T, Mn_T, Zn_T, NO_3-N and media temperature. Each experimental set started with the same spore suspension will be referred to as a series. A culture within that series investigating a particular parameter will be labeled by series letter and Aquil formulation number (e.g. culture C3 in Series C used the Aquil formulation prepared third).

Series A.

Previous research in Macrocystis gametophyte culturing indicated a substrate (e.g. microslides or coverslips) upon which spores could settle and adhere. Slides or slips could then be removed from the culturing media for examination. In preparation for trace element experiments, each microslide would require acid washing and immersion in Aquil to reduce micronutrient adsorption while culturing. If gametophytic development could occur with gametophytes suspended in culturing media (i.e., no substrate provided), then: 1) microslide preparation could be eliminated, and 2) suspended gametophytes could be quickly sampled with a Teflon pipette.

Series A tested the substrate requirement for gametophytic development by means of suspension cultures. Spore suspensions were injected into aerated FEP-Teflon culturing

vessels containing 250 mL Aquil. Suspensions were exposed only to TFE- and FEP-Teflon surfaces to which spores did not adhere.

Ultimately, gametophytes attached to each other in clusters sometimes exceeding 100 cells. Gametes were produced after seven culturing days from peripheral cells only. Interior cells exhibited little or no development. Dense bacterial growth appeared around the clusters after eight days (Figure 5.3). Previously healthy peripheral gametophytes became penetrated by bacteria after nine days. No embryonic sporophytes were produced. Suspension cultures were not a viable alternative to microslide cultures: use of the trough system was resumed.

Series B.

Effects on gametophytic development of adding nickel (at 100 nM) and increasing the copper concentration were examined in Series B. Time required for 50% of the daily sampled cells (10 per culture) to undergo gametogenesis was used to indicate development. Results from Series B (Table 5.2) demonstrated:

1. 100 nM Ni_T added to basal medium had no effect on gametogenesis;
2. 15 nM Cu_T inhibited gametogenesis and caused cell wall blistering; and
3. 100 nM nickel added with 15 nM copper reduced copper inhibition.

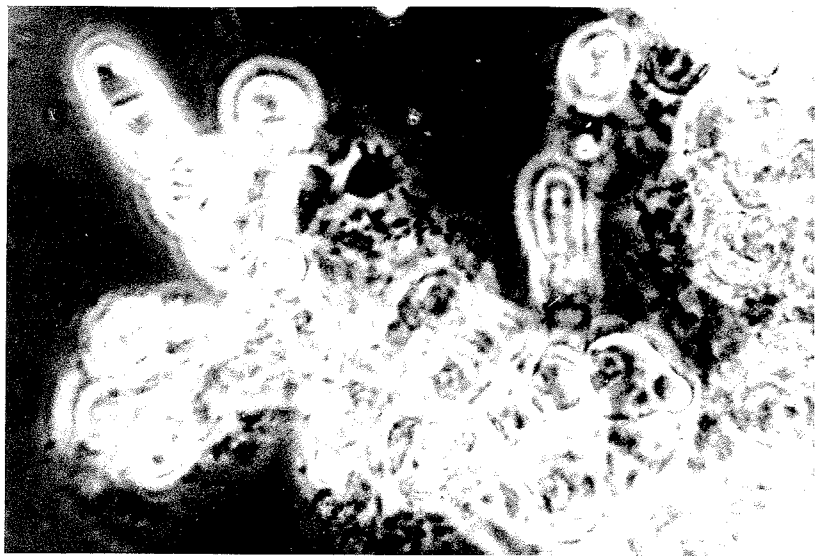


Figure 5.3. Peripheral gametophytes in Series A spore suspension cluster. Illustrates bacterial growth around gametophytes after eight culturing days.

Table 5.2. Series B Results

Sporophyll Source: Cameo Shores, Corona del Mar

Culturing Period: 5/10 - 5/24/78

Aquil Media:

B1 - Control

Co - (8.3, 11.4)[1]	MoO_4 - (7.3, 7.3)
Cu - (8.3, 13.9)	I - (7.3, 7.3)
Fe - (7.3, 20.2)	NO_3 - (4.5, 4.5)
Mn - (8.3, 9.4)	PO_4 - (5.7, 9.5)
Zn - (7.3, 10.7)	EDTA - (5.1, 13.0)

Treatment	Response Time, days[2]	Remarks
B1 - Control	10	Vegetative cultures, lacking pigment.
B2 - Cu augmentation - (7.8, 13.4)	14	Blistering of cell walls.
B3 - Ni addition - (7.3, 12.7)	10	No distinct difference from control.
B4 - Cu augmentation + Ni addition as above	12	Slight mitigation of Cu toxicity.

[1]Concentrations given in p units in the following format, (analytical concentration, computed free-ion concentration).

[2]Response parameter for Series B and C was the number of days required for 50% of the randomly sampled gametophytes to undergo gametogenesis (n=10).

Series C.

Selenite addition, copper omission and increased nitrate concentration were tested in Series C. Results (Table 5.3) indicated that:

1. selenite addition at 10 nM strongly inhibited gametophytic development;
2. copper omission retarded development only slightly; and
3. 50 μM NO_3-N did not stimulate developmental rate above that observed for 30 μM NO_3-N control cultures.

A culture using unenriched Monterey Bay surface water was included in Series C which required 13 days for gametogenesis. A temperature gradient along the culture trough stimulated bacterial contamination in warmer media near the trough drain (see Chapter 4). Four slips nearest each trough drain were therefore discarded after seven culturing days. Remaining slips were shifted towards the trough inlet, and thereby saved from further contamination. The four discarded slips were replaced with seven barren slips. Any temperature gradient effects were therefore experienced by all four trough cultures.

Series D.

Beginning with Series D, mean gametophytic length was used as our criterion of performance instead of the number of days required for gametogenesis (see Chapter 4). Series D through H cultures were run at 12.5 ±0.05°C. Decreasing

Table 5.3. Series C Results

Sporophyll Source: Lion's Head, Santa Catalina Island and Otter Point, Monterey Bay.

Culturing Period: 6/23 - 7/6/78, 6/29 - 7/12/78.

Aquil Media:

C1 - Control

Co - (8.0, 11.1)[1]	MoO_4 - (7.0, 7.0)
Cu - (8.0, 13.6)	I - (7.0, 7.0)
Fe - (7.0, 19.9)	NO_3 (4.5, 4.5)
Mn - (8.0, 9.1)	PO_4 (5.7, 9.5)
Zn - (7.0, 10.4)	EDTA - (5.1, 13.0)

Treatment	Response Time, days[2]		Remarks
C1 - Control	11	11[3]	Healthy, more deeply pigmented than Series B control.
C2 - SeO_3 addition - (8.0, 8.5)	-	-	Culture terminated, high mortality, contamination, slow development
C3 - Cu omission	13	13	Healthy, development slightly retarded.
C4 - NO_3 augmentation - (4.3, 4.3)	11	11	Vegetative growth, deep golden color.
C5 - Monterey Bay kelp bed seawater sample.		13	High bacterial contamination.

[1]Concentrations given in p units in the following format, (analytical concentration, computed free-ion concentration).

[2]Response parameter for Series B and C was the number of days required for 50% of the randomly sampled cells to undergo gametogenesis (n=10).

[3]Series C was repeated. Culture C5 was added to the series using Monterey Bay surface water as the culturing medium.

the nitrate concentration (from 30 to 20 μM) had no significant effect on lengths achieved. Differences between mean lengths in control cultures D1-D4 and in decreased nitrate cultures D5-D8 were not statistically significant at the 95% confidence level (Table 5.4).

Series E through H.

This group of experiments established optimal nutrient ranges for six elements (nM): Co_T = (20, 100), Cu_T = (0, 15), Fe_T = (100, 1000), Mn_T = (0, 30), Zn_T = (0, 300), and NO_3-N_T = (4000, 20,000) (Tables 5.5-5.8).

Series J.

A 2^3 full factorial experimental design examined effects from varying iron, zinc and media temperature on gametophytic growth (Figure 5.4 and Tables 5.9 and 5.10). Cultures J1 to J4 and J5 to J8 were subjected to media temperatures of 18°C and 14°C respectively. Two concentrations of iron and zinc were used; 100 and 400 nM, and 0 and 250 nM respectively. Other nutrient variables were held constant (Table 5.11). Four microslide replicates were employed per treatment.

Caltech Computing Center library subroutine LSQENP was used to fit results (Table 5-12) to the model: $Y = B_o + B_1x_1 + B_2x_2 + B_3x_3 + B_4x_1x_2 + B_5x_1x_3 + B_6x_2x_3 + B_7x_1x_2x_3$, where Y represented gametophyte length in microns and x_1, x_2 and x_3 represented normalized iron, zinc and temperature levels respectively.

Table 5.4. Series D Results

Sporophyll Source: Divers' Cove, Laguna Beach

Culturing Period: 7/13 - 7/27/78

Aquil Media:

D1-D4 - Control

Co - (8.0, 11.1)[1]	MoO_4 - (7.0, 7.0)
Cu - (8.0, 13.6)	I - (7.0, 7.0)
Fe - (7.0, 19.9)	NO_3 - (4.5, 4.5)
Mn - (8.0, 9.1)	PO_4 - (5.7, 9.5)
Zn - (7.0, 10.4)	EDTA - (5.1, 13.0)

Treatment		Mean length and 95% confidence interval μ, (n=10)	Significance[2]
D1	Control	196 ± 16	
D2		197 ± 16	
D3		186 ± 17	
D4		197 ± 29	
D5	NO_3 Reduction - (4.7,4.7)	185 ± 21	
D6		209 ± 20	
D7		179 ± 20	0
D8		164 ± 28	

[1]Concentrations given in p units in the following format, (analytical concentration, free ion concentration).

[2]Symbols denote that effect was:
++ = stimulatory at 95% confidence level,
+ = stimulatory at 90% confidence level.
0 = not statistically significant,
- = inhibitory at 90% confidence level,
-- = inhibitory at 95% confidence level.

Table 5.5. Series E Results

Sporophyll Source: Otter Point, Monterey Bay

Culturing Period: 7/19 - 8/2/78

Aquil Media:

E1 - Control

Co - (8.0, 11.1)[1]		MoO_4 -	(7.0, 7.0)
Cu - (8.0, 13.6)		I -	(7.0, 7.0)
Fe - (7.0, 19.9)		NO_3 -	(4.5, 4.5)
Mn - (8.0, 9.1)		PO_4 -	(5.7, 9.5)
Zn - (7.0, 10.4)		EDTA -	(5.1, 13.0)

Treatment	Mean length and 95% confidence interval p, (n=10)	Significance[2]
E1 - Control	171 ± 26	
E2 - Fe omission	73 ± 13	--
E3 - Mn omission	59 ± 9	

[1] Concentrations given in p units in the following format, (analytical concentration, free ion concentration).

[2] Symbols denote that effect was:
++ = stimulatory at 95% confidence level,
+ = stimulatory at 90% confidence level,
0 = not statistically significant,
- = inhibitory at 90% confidence level,
-- = inhibitory at 95% confidence level.

Table 5.6. Series F Results

Sporophyll Source: Divers' Cove, Laguna Beach

Culturing Period: 10/18 - 11/1/78

Aquil Media:

F1 - Control

Co - (8.0, 10.2)[1]	MoO_4 - (7.0, 7.0)
Cu - (8.0, 12.7)	I - (7.0, 7.0)
Fe - (7.3, 19.2)	NO_3 - (5.1, 5.1)
Mn - (8.0, 8.6)	PO_4 - (5.7, 9.5)
Zn - (7.0, 9.4)	EDTA - (6.0, 14.0)

Treatment	Mean length and 95% confidence interval μ, (n=10)	Significance[2]
F4 - Augmentation - Fe - (6.7, 19.2) EDTA - (5.4, 13.4)	172 ± 15	++
F3 - Cu reduction - (8.5, 13.1)	160 ± 17	+
F1 - Control	136 ± 11	0
F6 - Zn omission	116 ± 9	
F5 - Mn augmentation - (7.5, 8.1)	112 ± 15	-
F2 - Co omission	110 ± 14	
F7 - NO_3 reduction - (5.4, 5.4)	80 ± 9	--
F8 - AsO_4 addition - (7.0, 9.8)	56 ± 15	

[1]Concentrations given in p units in the following format, (analytical concentration, free ion concentration).

[2]Symbols denote that effect was:
++ = stimulatory at 95% confidence level,
+ = stimulatory at 90% confidence level,
0 = not statistically significant,
- = inhibitory at 90% confidence level,
-- = inhibitory at 95% confidence level.

Table 5.7. Series G Results

Sporophyll Source: Divers' Cove, Laguna Beach

Culturing Period: 11/8 - 11/22/78

Aquil Media:

G1 - Control

Co - (7.7, 11.1)[1]	MoO_4 - (7.0, 7.0)
Cu - (8.1, 14.0)	I - (7.0, 7.0)
Fe - (6.5, 19.7)	NO_3 - (4.8, 4.8)
Mn - (7.7, 9.0)	PO_4 - (5.7, 9.5)
Zn - (7.3, 10.4)	EDTA - (4.8, 13.2)

Treatment	Mean length and 95% confidence interval μ, (n=10)	Significance[2]
G6 - Zn augmentation - (6.3, 10.0)	164 ± 17	++
G2 - Co augmentation - (7.3, 10.7)	146 ± 12	+
G1 - Control	125 ± 9	0
G3 - Cu reduction - (9.0, 14.9)	107 ± 13	
G7 - NO_3 reduction (5.1, 5.1)	102 ± 16	-
G4 - Fe augmentation - (6.0, 19.1)	99 ± 15	
G8 - MoO_4 omission	92 ± 14	--
G5 - Mn reduction - (9.0, 10.3)	85 ± 14	

[1]Concentrations given in p units in the following format, (analytical concentration, free ion concentration).

[2]Symbols denote that effect was:
++ = stimulatory at 95% confidence level,
+ = stimulatory at 90% confidence level,
0 = not statistically significant,
- = inhibitory at 90% confidence level,
-- = inhibitory at 95% confidence level.

Table 5.8. Series H Results

Sporophyll Source: Divers' Cove, Laguna Beach

Culturing Period: 12/6 - 12/20/78

Aquil Media:

H1 - Control

Co - (7.4, 10.3)[1]	MoO_4 - (7.0, 7.0)
Cu - (8.3, 13.7)	I - (7.0, 7.0)
Fe - (6.5, 19.1)	NO_3 - (4.8, 4.8)
Mn - (7.7, 8.6)	PO_4 - (5.7, 9.5)
Zn - (6.5, 9.7)	EDTA - (5.3, 13.3)

Treatment	Mean length and 95% confidence interval μ, (n=10)	Significance[2]
H6 - Zn reduction - (7.0, 10.2)	193 ± 13	+
H1 - Control	173 ± 9	0
H7 - NO_3 reduction - (5.1, 5.1)	160 ± 18	
H3 - Cu augmentation - (7.7, 13.1)	147 ± 15	--
H2 - Co augmentation - (7.9, 9.8)	131 ± 17	
H8 - I omission	95 ± 21	
H5 - Mn reduction - (8.3, 9.2)	103 ± 14	
H4 - Fe reduction - (7.0, 19.6)	88 ± 14	

[1]Concentrations given in p units in the following format, (analytical concentration, free ion concentration).

[2]Symbols denote that effect was:
++ = stimulatory at 95% confidence level,
+ = stimulatory at 90% confidence level,
0 = not statistically significant,
- = inhibitory at 90% confidence level,
-- = inhibitory at 95% confidence level.

Figure 5.4. Series J, a 2^3 full factorial experiment. Design points are formatted such that a(b,c,d) denotes culture number a at x_1, x_2, x_3 normalized levels b, c, d respectively.

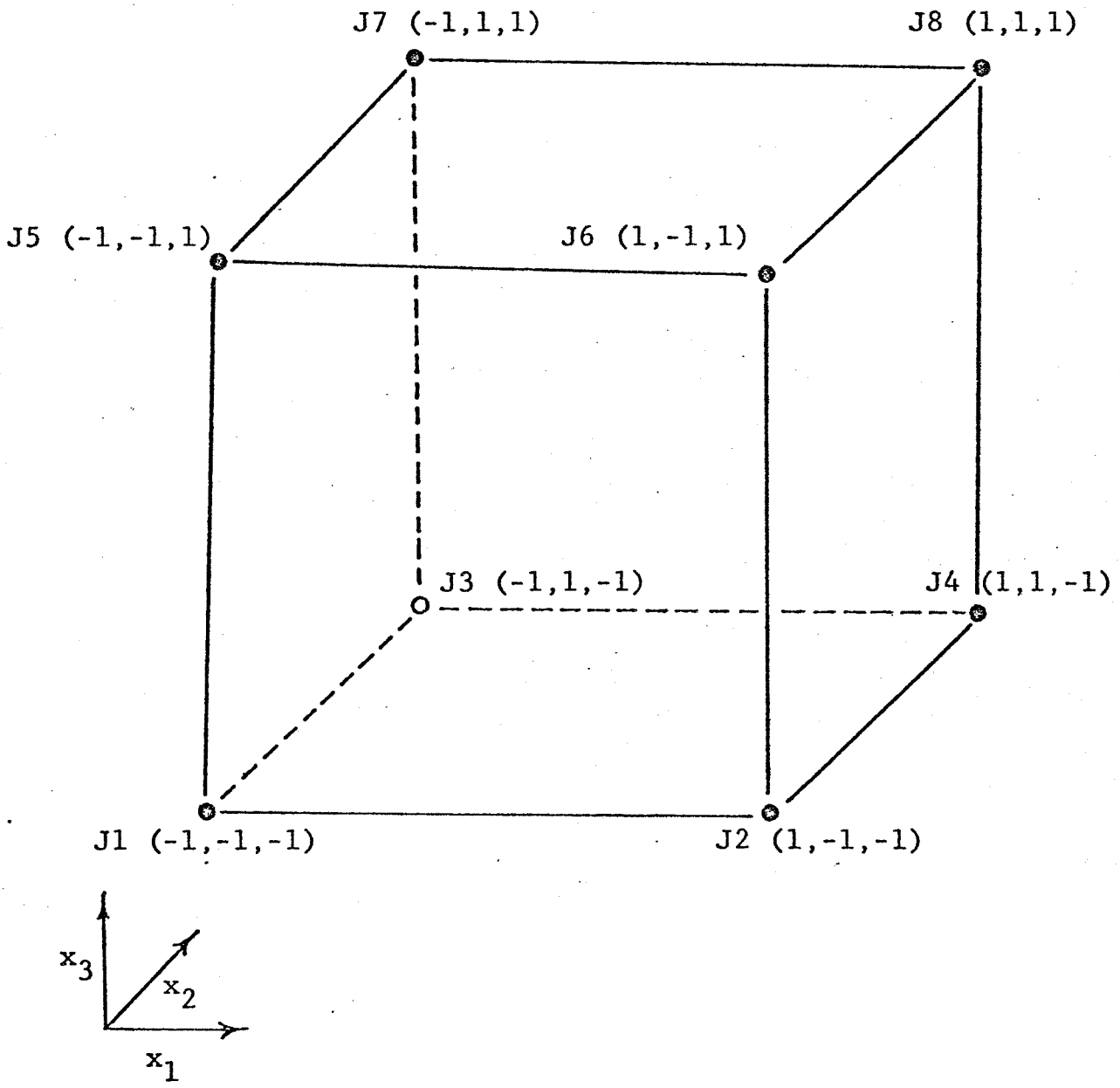


Table 5.9. Normalized variables defined for Series J

Variable/Level	-1	1
x_1 (Fe_T conc.)	(7.0, 19.7)[1]	(6.4, 19.2)
x_2 (Zn_T conc.)	-	(6.6, 10.0)
x_3 (Temperature)	18°C	14°C

Table 5.10. Series J culture treatments. Four microslide replicates were employed per treatment.

	Treatment		
Culture	x_1	x_2	x_3
J1	-1	-1	-1
J2	1	-1	-1
J3	-1	1	-1
J4	1	1	-1
J5	-1	-1	1
J6	1	-1	1
J7	-1	1	1
J8	1	1	1

[1]Concentrations given in p units in the following format, (analytical concentration, computed free-ion concentration).

Table 5.11. Nutrient variables held constant in Series J and K.

Nutrient	Analytical concentration, (nM)	Computed free-ion concentration, (nM)	Major species (%)
Metals			
Co^{2+}	40	0.04	CoEDTA (99)
Cu^{2+}	5	$2x10^{-5}$	CuEDTA (99)
Mn^{2+}	10	1	MnEDTA (66) $MnCl^{+}$ (22)
Ligands			
MoO_4^{2-}	100	100	Free ion (100)
$EDTA^{2-}$	$6x10^{3}$	$7x10^{-5}$	CaEDTA (ca. 90)*
NO_3^{-}	$1.5x10^{4}$	$1.5x10^{4}$	Free ion (100)
PO_4^{-}	$2x10^{3}$	0.3	HPO_4^{-} (51) $MgHPO_4$ (47)
I^{-}	100	100	Free ion (100)

* Percentage varied slightly depending on iron and zinc levels.

Table 5.12. Series J Results

Culture Number	Mean length and 95% confidence interval μ, (n=40)
J1	41 ± 5
J2	33 ± 5
J3	31 ± 4
J4	25 ± 4
J5	107 ± 6
J6	121 ± 6
J7	110 ± 7
J8	122 ± 6

The temperature effect was dominant (Table 5.13). Bacterial infestation was observed in 18°C cultures. Gametophytic density was an order of magnitude lower in 18°C cultures than in 14°C cultures. Gametophytes that did survive the 14-day culturing period at 18°C had not developed beyond first appearance of plastids, and their color was poor to completely absent. Cultures at 14°C were deep golden and discernably darker than gametophytes of the previous series. Multicellular sporophytes were observed in abundance after 14 culturing days in culture J8. Cultures J5 and J6 (no Zn added) produced fewer gametophytes than J8, but those surviving appeared healthy. Only four embryonic sporophytes were observed through J5 and J6 cultures (one in J5 and three in J6). Gametophytic survival among the 14°C culture group was poorest in J7 (100 n$\underline{M}$ Fe_T, 250 n$\underline{M}$ Zn_T). Gametophytes which survived 14 days in J7 were more pale than those of cultures J5, J6 and J8. The fact that iron stimulated growth at 14°C but not at 18°C resulted in a significant iron-temperature interactive term ($p < 0.01$). Zinc-temperature interaction was less pronounced but still significant at $p < 0.05$.

Table 5.13. Series J analysis of variance.

Model: $Y = 73 - 41x_3 - 5x_1x_3 - 3x_2x_3$,

where: Y = mean gametophytic length in microns,

x_1= normalized Fe_T (as defined previously),

x_2= normalized Zn_T, and

x_3= normalized media temperature.

Variation due to:	df	SS	MS	F ratio
Main effects				
x_3	1	273076	273076	953**
First order interactions				
x_1x_3	1	4101	4101	14**
x_2x_3	1	1156	1156	4*
Lack of fit	4	969		
Within subgroup	152	43741		
Error	156	44710	287	
Total	159	323043		

*Effect significant at 95% confidence level ($F_{0.95(1,156)}=3.91$).

*Effect significant at 99% confidence level ($F_{0.99(1,156)}=6.80$).

CHAPTER 6

EXPLORING THE OPTIMAL REGION

Series I.

Effects of suboptimal nitrate concentrations (1, 5, 9, and 13 μM) and media temperatures (10°C and 14°C) on gametophytic growth were examined. Two microslide replicates were employed per treatment (Table 6.1).

Computing Center subroutine LSQENP provided a least squares fit of average gametophyte length to the equation $Y = A(1-e^{Bx})$, selected as a model of response by growth to a given nutrient under investigation. Y represents gametophyte length in microns and x represents micromolar nitrate concentration. A represents maximum gametophytic length in microns achieved at high NO_3 concentrations (>20 μM, see Chapter 5, Series D). B reflects the rate at which Y converges to A as a function of x (Table 6.2). Computer subroutine MAGIC was used to plot length data with LSQENP-generated curves (Figure 6.1). The non-linear model was selected because: 1) gametophytic length in response to increased nitrate concentration within the 0-50 μM range exhibited no significant change above 20 μM [NO_3] (see Chapter 5, Series C and D results), and 2) increased media temperatures increased maximum gametophytic length. Parameters A and B were significant at the 95% confidence level for both 10°C and 14°C cultures (Table 6.2). Correlation coefficients (r) of 0.92

Table 6.1. Series I Results.

Sporophyll source: Divers' Cove, Laguna Beach.

Culturing period: 3/6 - 3/20/79.

Aquil media:

I4 - Control

Co - (7.4, 10.2)[1]	MoO_4 - (7.0, 7.0)
Cu - (8.3, 13.6)	I - (7.0, 7.0)
Fe - (6.6, 19.1)	NO_3 - (4.9, 4.9)
Mn - (8.0, 8.9)	PO_4 - (5.7, 9.5)
Zn - (7.0, 10.1)	EDTA - (5.4,13.4)

Treatment	Mean length and 95% confidence interval μ, (n = 20)	Significance[2]
I4' - 14°C, Control	165 ± 5	0
I3' - 14°C, NO_3 decrease - (5.0,5.0)	160 ± 8	
I3 - 10°C, NO_3 decrease - (5.0, 5.0)	145 ± 9	--
I4 - 10°C, Control	144 ± 7	
Further nitrate decrease:		
I2' - 14°C, (5.3, 5.3)	124 ± 9	---
I2 - 10°C, (5.3, 5.3)	112 ± 8	
I1 - 10°C, (6.0, 6.0)	45 ± 6	
I1' - 14°C, (6.0, 6.0)	31 ± 6	

[1]Concentrations given in p units in the following format, (analytical concentration, computed free-ion concentration).

[2]Symbols denote that effect was:
++ = stimulatory at 95% confidence level,
+ = stimulatory at 90% confidence level,
0 = not statistically significant,
- = inhibitory at 90% confidence level,
-- = inhibitory at 95% confidence level,
---= inhibitory at 99.9% confidence level.

Table 6.2. Non-linear analysis of Series I data. Parameter values and 95% confidence intervals were calculated using Computing Center subroutine LSQENP (n=80).

Model: $Y = A(1-e^{Bx})$ where Y = mean gametophytic length in in microns, and x = nitrate concentration (μM).

Parameter	$10^{o}C$	$14^{o}C$
A	148 ± 13	177 ± 17
B	-0.32 ± 0.10	-0.23 ± 0.06
$\|r\|$	0.92	0.98

Table. 6.3. Comparison of predicted and observed mean gametophytic lengths at various nitrate concentrations and temperatures.

Culture	Observed mean length	Predicted length	% Error
Temperature = $10^{o}C$			
I1	45	41	-9
I2	112	118	5
I3	145	140	-3
I4	144	146	1
Temperature = $14^{o}C$			
I1'	31	38	23
I2'	124	124	0
I3'	160	157	-2
I4'	165	169	2

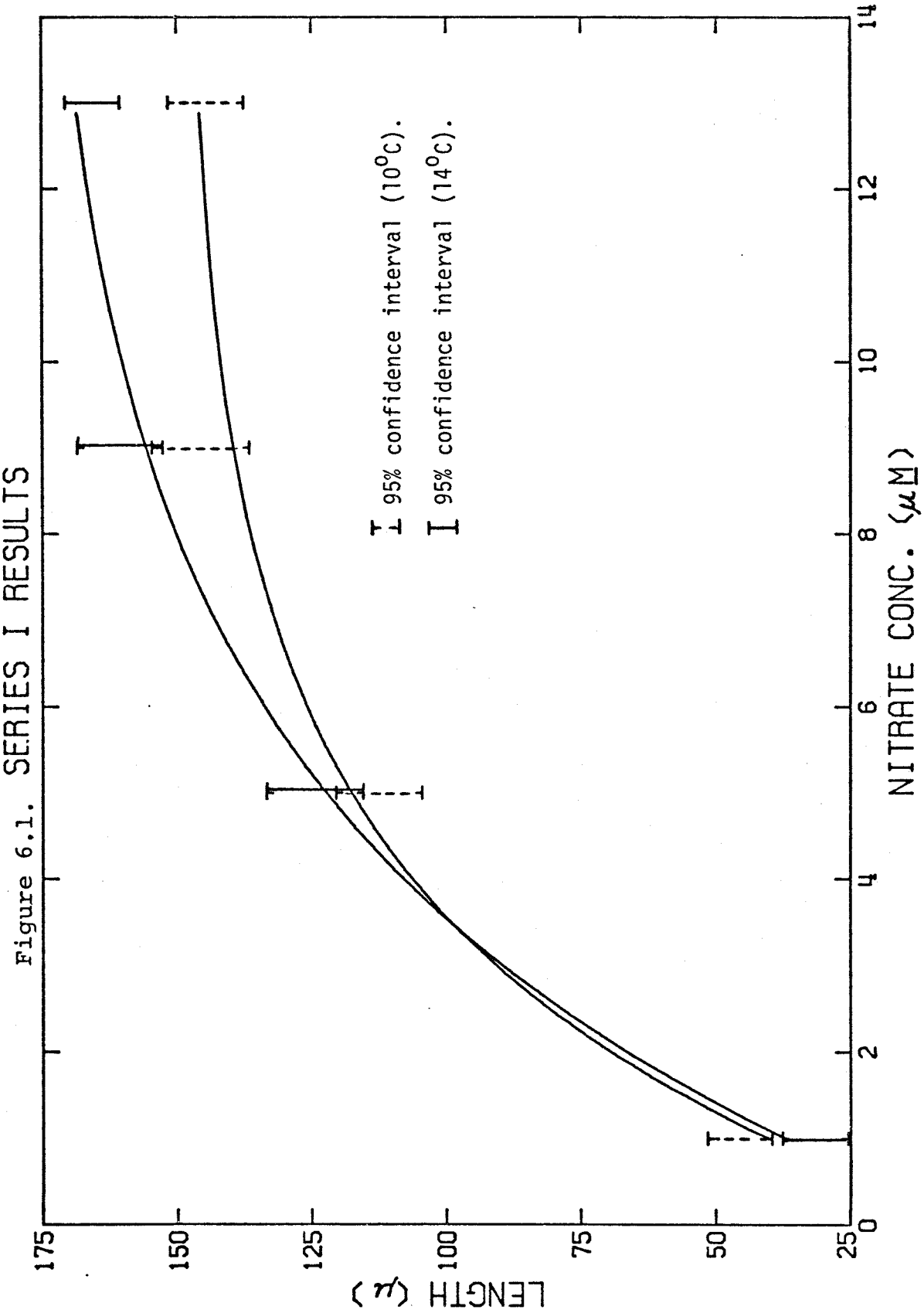

SERIES I RESULTS
95% confidence interval (10°C).
95% confidence interval (14°C).
LENGTH (μ)
NITRATE CONC. (μM)
175
150
125
100
75
50
25
0
2
4
6
8
10
12
14

Figure 6.1.

and 0.98 were determined for 10°C and 14°C cultures respectively. Y values calculated with the LSQENP-generated equation compared well with observed data (Table 6.3).

Aquil samples I1' and I4' were analyzed for NO_3+NO_2-N before and after the 3-day cycle of renewing media (Table 6.4). Nitrate disappearances in I4' indicated an uptake rate of $3x10^{-7}$moles-N/day or $2x10^{-12}$ moles-N/cell-day. Assuming a rod-shaped cell with R_c = 10 μ and l_c = 170 μ, estimated from I4' gametophytes after 14 culturing days, cell volume = $5x10^{-8}$ cm^3. I4' produced nearly maximum gametophytic growth. Therefore estimated I4' cell nitrate uptake may correspond to Michaelis-Menten maximum velocity (V_{max}). I4' cell uptake rate was converted to uptake rate per unit mass and V_{max} was estimated by assuming that water density corresponded approximately to protoplasmic density: $2x10^{-2}$ $\frac{\text{moles-N}}{\text{cell-day}}$ (day/24 hr) $(\frac{\text{cell}}{5x10^{-8}cm^3})(cm^2/g)$ = 2 μmoles-N/g(wet)-hr. A Michaelis-Menten constant (K_s) was estimated: $(1-e^{Bx})$ = ½ when B_x = -0.69. B = -0.24 μM^{-1}. Therefore, x = 0.69/0.24 = 3 μM. V_{max} and K_s values of 3.05 μmoles-N/g(wet)-hr and 13.1 μM respectively were determined for Macrocystis juvenile sporophytes (Haines and Wheeler, 1978). The lower V_{max} determination for Macrocystis gametophytes than for juvenile sporophytes may have resulted from using 14-day I4' gametophytic dimensions with nitrate disappearances over a 3-day period. (Experiments by Haines and Wheeler (1978) examined

Table 6.4. Nitrate analysis (μM) of Aquil from cultures I1' and I4'.

Sample	Sampling dates	Mean concentration and 95% confidence interval (n=3)	Spiking level
I1'(before)	3/11,14,17	0.84 ± 0.22	1.00
I1'(after)	3/14,17,20	0.41 ± 0.31	1.00
I4'(before)	3/11,14,17	13.44 ± 0.76	13.00
I4'(after)	3/14,17,20	9.22 ± 0.67	13.00

nitrate uptake over a 100-minute duration). Growth of I4' gametophytes between day 11 and day 14 suggest that a gametophyte volume estimate lower than $5x10^{-8}cm^3$ may have been appropriate. Differences in K_s values indicate that:

1. Uptake sites for gametophytes have a higher affinity for nitrate than uptake sites for juvenile sporophytes, or
2. The convergence to a gametophytic growth maximum observed in Series I cultures with increased nitrate does not correspond to saturated nitrogen uptake (rigorous proof supporting this correspondence is lacking).

Series K.

A 3^2 factorial experiment examined effects on Macrocystis gametophytic growth from varying iron and zinc levels in Aquil within their optimal ranges (Figure 6.2). Design corner points K6 to K9 corresponded to points J5 to J8 from the 2^3 experiment previously discussed (see Chapter 5, Series J). Effects on gametophytic growth due to different sporophyll batches could therefore be assessed. Iron at 100, 250 and 400 nM, and zinc at 0, 125 and 250 nM were used (Table 6.5). Concentrations of other nutrients were held constant in Series K at levels used in Series J (see Table 5.11). Four microslide replicates were employed per treatment.

Figure 6.2. Series K, a 3^2 factorial experiment where x_1 and x_2 are normalized Fe_T and Zn_T concentrations respectively. Design points are symbolized such that a(b,c) denotes culture number a at x_1, x_2 normalized levels represented by the symbols b, c respectively as defined in Table 6.5.

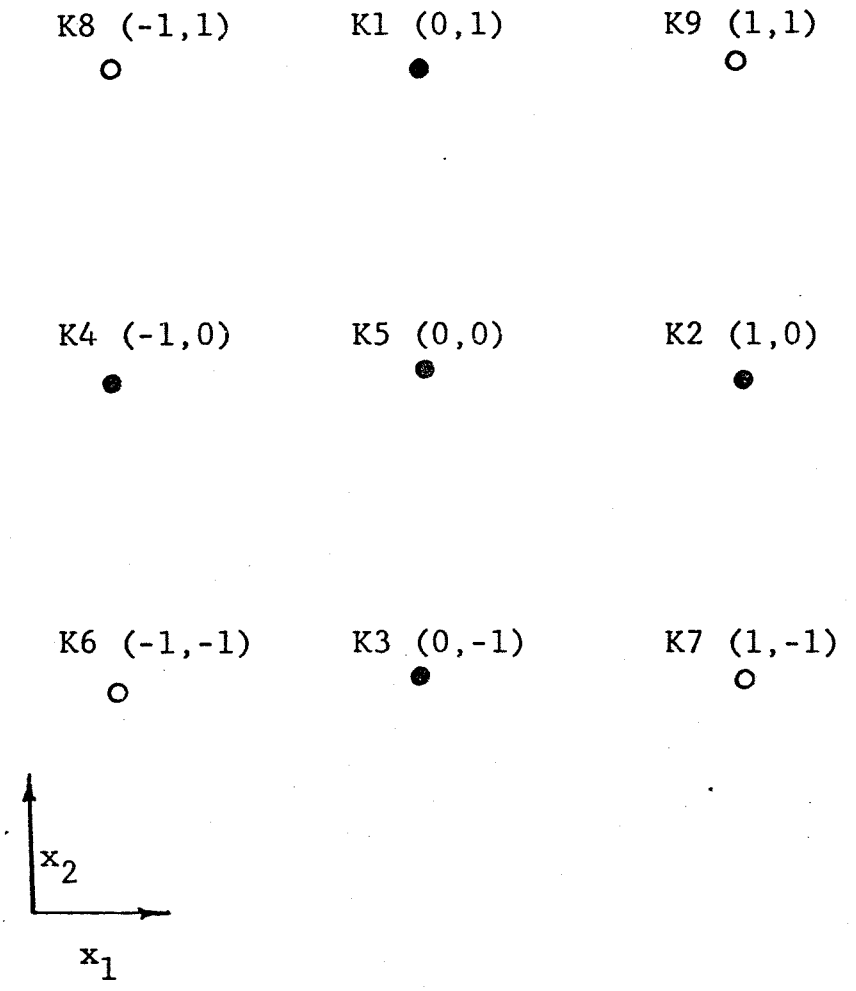


Table 6.5. Normalized variable concentrations defined for Series K. Concentrations given in p units in the following format, (analytical concentration, computed free-ion concentration). The symbols representing each concentration are also shown.

Variable	Symbols -1	0	1
		Concentrations	
x_1 (Fe_T conc.)	(7.0, 19.7)	(6.6, 19.4)	(6.4, 19.2)
x_2 (Zn_T conc.)	-	(6.9, 10.2)	(6.6, 10.0)

Media temperatures were set at 14°C. Sporophylls collected off Lion's Head, Santa Catalina Island were used after repeated efforts failed to produce a spore suspension from Laguna Beach sporophylls. Producing spore suspensions from Laguna Beach sporophylls was difficult following upwelling periods. Sporophylls exposed to cold temperatures associated with upwelled water (<10°C) had presumably been forced to release all their mature spores prior to collection. Sporophylls collected off Catalina Island's eastern shore were apparently less affected by upwelling processes. This source was accordingly used when sporophylls from the mainland were not satisfactory.

Average length data (Table 6.6) were fit to the model:

$$Y = \sum_{j=0}^{2} \sum_{i=0}^{2} B_{ij} x_1^{\,i} x_2^{\,j}$$ using Caltech Computing Center Library subroutine LSQENP, where Y equaled gametophytic length in microns, and x_1 and x_2 were normalized iron and zinc total concentrations respectively, defined in Table 6.5. LSQENP generated least squares estimates for coefficients Bij (Table 6.7). Maximum gametophytic length was determined at Fe_T = 340 n$\underline{M}$ and Zn_T - 135 n$\underline{M}$. Predicted length at these concentrations is 139 microns (Table 6.8) based on the LSQENP-generated equation.

Multicellular embryonic sporophytes were observed in abundance after 12 culturing days in culture K5. Embryonic

Table 6.6. Series K results. Culture treatments shown by means of symbols (defined in Table 6.5) represent the normalized variable concentrations (x_1, x_2).

Sporophyll source: Divers' Cove, Laguna Beach.

Culturing period: 5/17 - 5/31/79.

Culture	Treatment	Mean length and 95% confidence interval, μ (n=40)
K1	(0,1)	119 $\pm$ 5
K2	(1,0)	137 $\pm$ 5
K3	(0,-1)	119 $\pm$ 5
K4	(-1,0)	121 $\pm$ 5
K5	(0,0)	139 $\pm$ 4
K6 = J5	(-1,-1)	116 $\pm$ 7
K7 = J6	(1,-1)	111 $\pm$ 6
K8 = J7	(-1,1)	105 $\pm$ 5
K9 = J8	(1,1)	122 $\pm$ 6

Table 6.7. Series K analysis of variance.

Model: $Y = 137 + 8.0x_1 + 5.5x_1x_2 - 7.0x_1^2 - 17.0x_2^2 - 5.0x_1x_2^2$.

where x_1 = normalized Fe_T concentration, and

x_2 = normalized Zn_T concentration.

Variance due to	df	SS	MS	F Ratio
Main Effects				
x_1	1	4969	4969	15.4**
Quadratic Effects				
x_1^2	1	3809	3809	11.8**
x_2^2	1	24012	24012	74.2**
First Order Interaction				
x_1x_2	1	4995	4995	15.4**
Higher Order Interactions				
$x_1x_2^2$	1	1449	1449	4.5*
Within Subgroup	351	114136		
Lack of Fit	3	418		
Error	354	114554		
Total	139	153788		

*Effect significant at 95% confidence level ($F_{0.95(1,354)} = 3.87$).

**Effect significant at 99% confidence level ($F_{0.99(1,354)} = 7.72$).

Table 6.8. Comparison between observed mean gametophytic lengths for Series K and lengths determined by LSQENP-generated equation (referred to as predicted lengths).

Culture	Observed mean length, μ	Predicted length, μ	%Error
K1	119	120	1
K2	137	138	1
K3	119	120	1
K4	121	122	1
K5	139	137	-2
K6	116	115	-1
K7	111	110	-1
K8	105	104	-1
K9	122	121	-1

*Predicted maximum = 139 microns at Fe_T = 340 n$\underline{M}$, Zn_T = 135 n$\underline{M}$.

sporophytes were also observed in cultures K2 and K9 after 14 days but were much fewer than in K5, as well as being poorly pigmented and surrounded by bacterial clusters. Thus K5 was clearly the optimal medium in this experimental series. After 14 days, even a few K5 sporophytes began to lose pigmentation and cell wall integrity. Blistering among gametophytes and sporophytes and bacterial growth around unhealthy plants were observed. Data did not reflect this sudden change because gametophytes were measured before these problems affected gametophytic length.

Nutrient demands apparently increased with time as gametophytic size increased (over 100 fold during the 14-day culturing period). Batch culturing may have resulted in depleted nutrients in the period immediately preceding media exchanges. Depletion may have become more pronounced when biomass increased sharply after sporophytes were produced. Copper depletion was not a problem, as indicated in atomic absorption spectrophotometric (AAS) analysis of Aquil before and after media exchanges. Depletion of other micronutrients, however, may have occurred. If culturing periods in excess of 14 days are desired, larger culturing vessel volume or increasing the frequency of media exchanges should be considered.

Differences in gametophytic lengths between Series J and K were not significant at a 95% confidence level (Table

Table 6.9. Length comparisons between Series J and Series K cultures. Mean gametophytic lengths and 95% confidence intervals given in microns (n=40). The two series were initiated with sporophylls collected from different Macrocystis adult sporophytes on different days (23 April 1979 for Series J and 17 May 1979 for Series K.

Cultures	Series J	Series K
J5 = K6	107 ± 6	116 ± 7
J6 = K7	121 ± 6	111 ± 6
J7 = K8	110 ± 7	105 ± 5
J8 = K9	122 ± 6	122 ± 6

6.9). The fact that sporophylls had been collected from different Macrocystis adult sporophytes on different days for Series J and K (23 April and 17 May, 1979 respectively) was thus of no importance.

Series Y - Omission Cultures.

The essential nature was examined of eight nutrient variables present in the Series K-derived Aquil formulation (Table 6.9a). Nitrogen, the ninth nutrient variable was not tested because: 1) Series I had determined that reduction of available nitrogen from 13 μM to 1 μM affected gametophytic growth drastically (mean length and 95% confidence intervals from 14 days in 13 μM NO_3 were 165±5 μ; mean length from 14 days in 1 μM NO_3 was 31±7 μ, t=17.4: difference significant at $p << 0.001$, 38 df), 2) the need for this element in protein, nucleic acid, and chlorophyll synthesis is obvious, and 3) the culturing system was only capable of handling eight nutrient variables per culturing series. Omissions of any of the eight nutrient variables from the successful Aquil formulation led to significantly reduced mean lengths relative to the control and to failure of gametogenesis and sporophyte production (Table 6.10). Gametophytes in no-Cu, no-Zn, and no-Co media were clearly still vegetative after 14 days but might have proceeded to gametogenesis if transferred to a complete medium. Perhaps vegetative growth only requires minute, undetectable levels of copper and zinc (i.e. <1 nM) while gametogenesis may impose higher demands for the two

Table 6.9a. Nanomole/l of nine nutrient variables and of EDTA added to an Aquil medium that sustained development by Macrocystis zoospores in batch cultures, through the gametophytic stage to embryonic sporophytes.

Nutrient	Analytical concentration, nM	Computed free-ion concentration, nM	Major species (%)
Metals			
Fe^{3+}	350	$7x10^{-11}$	FeEDTA (100)
Mn^{2+}	10	1	MnEDTA (65)
			$MnCl^{+}$ (23)
Co^{2+}	50	0.05	CoEDTA (99)
Cu^{2+}	5	$2x10^{-5}$	CuEDTA (99)
Zn^{2+}	150	0.08	ZnEDTA (100)
Ligands			
MoO_4^{2-}	100	100	Free ion (100)
$EDTA^{2-}$	$6x10^{3}$	$7x10^{-5}$	CaEDTA (89)
			FeEDTA (6)
NO_3^{-}	$2x10^{4}$	$2x10^{4}$	Free ion (100)
PO_4^{3-}	$2x10^{3}$	0.3	HPO_4^{2-} (51)
			$MgHPO_4$ (47)
I^{-}	100	100	Free ion (100)

Table 6.10. Series Y Results.

Sporophyll source: Cameo Shores, Corona del Mar.

Culturing period: 9/10 - 9/24/79.

Aquil media:

Y1 - Control

Co - (7.3, 10.3)	MoO_4 - (7.0, 7.0)
Cu - (8.3, 13.7)	I - (7.0, 7.0)
Fe - (6.5, 19.2)	NO_3 - (4.7, 4.7)
Mn - (8.0, 9.0)	PO_4 - (5.7, 9.5)
Zn - (6.8, 10.1)	EDTA - (5.2, 13.2)

Treatment	Mean length and 95%[2] confidence interval, μ (n=20)	Remarks
Y1 - Control	138 ± 7	Embryonic development observed.
Y3 - no Cu	119 ± 9*	Healthy, no sporophytes.
Y8 - no Zn	112 ± 10**	"
Y2 - no Co	103 ± 8***	"
Y5 - no I	85 ± 10***	Few surviving gametophytes.
Y7 - no Mn	60 ± 9***	"
Y6 - no MoO_4	64 ± 8***	"
Y4 - no Fe	65 ± 6***	"
Y9 - no PO_4	49 ± 6	"

[1]Concentrations given in p units in the following format, (analytical concentration, computed free-ion concentration).

[2]Asterisks *, **, *** denote that difference between control and test culture responses were significant at 95%, 99% and 99.9% respectively by Student's t-test (df = 38).

elements. Statistical significance of mean length differences between controls and test cultures were least for the no-Cu and no-Zn cultures ($p < 0.05$ and $p < 0.01$, respectively). Values of p were all less than 0.001 for the other six omission cultures. Improved performances in the no-Cu and no-Zn media conceivably might have resulted from introduction of undetectable levels of copper and zinc by airborne contaminants (Hidy, 1974) or from incomplete removal of impurities by the Chelex-100 columns. These results with Cu and Zn omissions illustrate the extreme difficulties that are encountered when one's goal is to produce media absolutely free from any kind of contamination. Complete freedom from contamination could not have been achieved in spite of the elaborate precautions taken in this research project.

Series L, M and N.

Primary and interactive effects from zinc, manganese, copper and cobalt on Macrocystis gametophytic growth and development were examined. Other Aquil nutrient variables were held constant (Table 6.11). A 3^{4-1} fractional factorial experiment was conducted over a 4-month period, 27 July to 27 November 1979 (Figure 6.3, Table 6.12). Three levels of each micronutrient parameter were used (Table 6.13). Four microslide replicates were employed per treatment. Because 108 microslide cultures were required, this experiment was performed in three blocks, called Series L, M and N (Table 6.13a). Media temperatures were fixed at $14^{o}C$,

Table 6.11. Aquil nutrients held constant in Series L, M and N.

Nutrient	Analytical concentration, (nM)	Computed free-ion concentration, (nM)	Major species (%)
Metals			
Fe^{3+}	350	$7x10^{-11}$	FeEDTA (100)
Ligands			
MoO_4^{2-}	100	100	Free ion (100)
$EDTA^{2-}$	$6x10^3$	$7x10^{-5}$	CaEDTA (89)
			FeEDTA (6)
NO_3^-	$2x10^4$	$2x10^4$	Free ion (100)
PO_4^{3-}	$2x10^3$	0.3	HPO_4^{2-} (51)
			$MgHPO_4$ (47)
I^-	100	100	Free ion (100)

Figure 6.3. Series L, M and N, a 3^{4-1} fractional factorial experiment. Design points are symbolized such that Ab(c) denotes Series A, culture number b at x_4 concentration c.

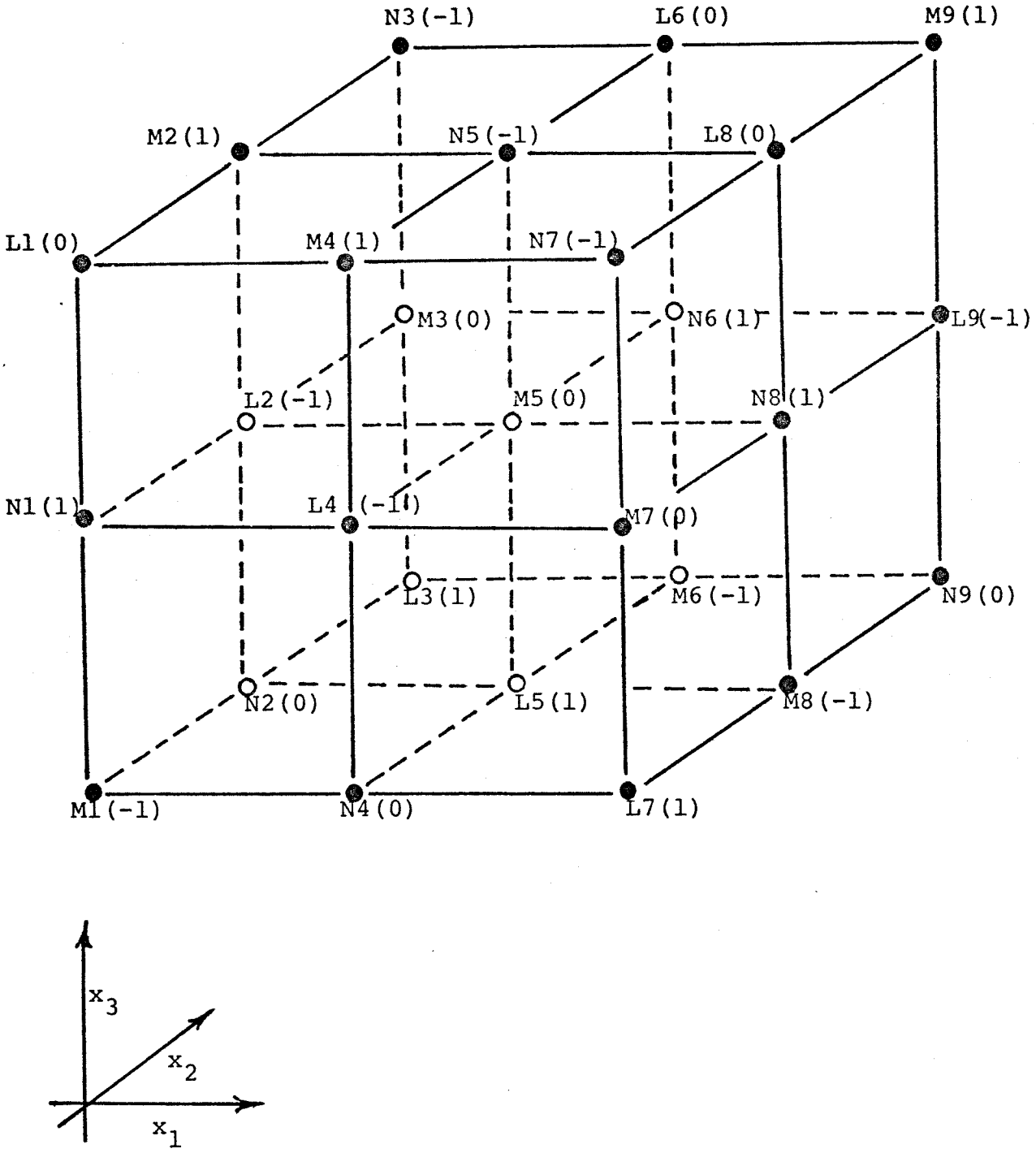


Table 6.12. Alias structure for the 3^{4-1} fractional factorial experiment. Three-way and higher interactions were assumed negligible.

Factor effects	df	Aliases (I = $x_1x_2x_3x_4$)
Main		
x_1	2	$x_1x_2^2x_3^2x_4^2 = x_2x_3x_4$
x_2	2	$x_1x_2^2x_3x_4 = x_1x_3x_4$
x_3	2	$x_1x_2x_3^2x_4 = x_1x_2x_4$
x_4	2	$x_1x_2x_3x_4^2 = x_1x_2x_3$
Two-way interactions		
x_1x_2	2	$x_1x_2x_3^2x_4^2 = x_3x_4$
x_1x_3	2	$x_1x_2^2x_3x_4^2 = x_2x_4$
x_2x_3	2	$x_1x_2^2x_3^2x_4 = x_1x_4$
$x_1x_2^2$	2	$x_1x_3^2x_4^2 = x_2x_3^2x_4^2$
$x_1x_3^2$	2	$x_1x_2^2x_4^2 = x_2x_3^2x_4$
$x_1x_4^2$	2	$x_1x_2^2x_3^2 = x_2x_3x_4^2$
$x_2x_3^2$	2	$x_1x_2^2x_4 = x_1x_3^2x_4$
$x_2x_4^2$	2	$x_1x_2^2x_3 = x_1x_3x_4^2$
$x_3x_4^2$	2	$x_1x_2x_3^2 = x_1x_2x_4^2$
Total	26	

Table 6.13. Concentrations of the normalized variables used for Series L, M and N*. and the symbols representing each concentration.

Variable	Symbols -1	0	+1
x_1 (Zn_T)	(8.0,11.3)	(6.8,10.1)	(6.5,9.8)
x_2 (Mn_T)	(8.3,9.3)	(7.6,8.6)	(7.3,8.3)
x_3 (Cu_T)	-	(8.0,13.5)	(7.7,13.2)
x_4 (Co_T)	(8.3,11.3)	(7.3,10.3)	(7.0,10.0)

Table 6.13a. Combinations of the normalized variables (x_1, x_2, x_3, x_4), used for each series of nine cultures, shown by means of the symbols defined in Table 6.13.

Culture No.	Series L (Block 1)	Series M (Block 2)	Series N (Block 3)
1	(-1,-1,1,0)	(-1,-1,-1,-1)	(-1,-1,0,1)
2	(-1,0,0,-1)	(-1,0,1,1)	(-1,0,-1,0)
3	(-1,1,-1,1)	(-1,1,0,0)	(-1,1,1,-1)
4	(0,-1,0,-1)	(0,-1,1,1)	(0,-1,-1,0)
5	(0,0,-1,1)	(0,0,0,0)	(0,0,1,-1)
6	(0,1,1,0)	(0,1,-1,-1)	(0,1,0,1)
	(1,-1,-1,1)	(1,-1,0,0)	(1,-1,1,-1)
8	(1,0,1,0)	(1,0,-1,-1)	(1,0,0,1)
	(1,1,1,1)	(1,1,0,-1)	(1,1,-1,0)

*Concentrations given in p units in the following format, (analytical concentration, free ion concentration).

Results of Series L, M and N (Tables 6.14-1,16) were fit to the equation:

$$Y = \sum_{i,j} \left(\sum_{l=0}^{2} \sum_{k=0}^{2} B_{kl} x_i^k x_j^l \right)$$

where: Y = mean gametophytic length in microns,

x_i and x_j = normalized micronutrient levels ($i=1,2,3,4$, $j=1,2,3,4$, $i \neq j$),

B_{kl} = the coefficient associated with x_i and x_j at integer powers k and 1 respectively. Caltech Computing Center Library subroutine LSQENP was used to determined least squares estimates for the B_{kl} coefficients.

The analysis of variance (Table 6.17) showed that eleven coefficients were significant at the 99.9% confidence level. The resulting model indicated optimal concentrations of 169, 28, 12 and 65 n$\underline{M}$ for Zn_T, Mn_T, Cu_T and Co_T respectively (Table 6.18). Predicted maximum length under these conditions was 138 microns. Observed mean lengths compared favorably with lengths determined by the LSQENP-generated equation (Tables 6.19-6.21). M5 was the only culture to produce embryonic sporophytes within 14 days after spore release among the 27 treatments of Series L, M and N (Figure 6.4).

Series P.

Series L, M and N employed a 3^{4-1} fractional factorial design to reduce experimental size by a third (i.e. 27

The following tables list gametophyte mean lengths and 95% confidence intervals for each treatment in the 3^{4-1} fractional factorial experiment executed in three blocks called Series L, M and N.

Table 6.14. Series L results. Culture treatments are shown as symbols (defined in Table 6.13) representing the normalized variable concentrations (x_1, x_2, x_3, x_4).

Sporophyll source: Cameo Shores, Corona del Mar.

Culturing period: 9/3 - 9/17/79.

Culture	Treatment	Mean length and 95% confidence interval, μ (n=40)
L1	(-1,-1,1,0)	58 ± 5
L2	(-1,0,0,-1)	96 ± 5
L3	(-1,1,-1,1)	85 ± 5
L4	(0,-1,0,-1)	77 ± 6
L5	(0,0,-1,1)	118 ± 6
L6	(0,1,1,0)	92 ± 6
L7	(1,-1,-1,1)	86 ± 6
L8	(1,0,1,0)	91 ± 5
L9	(1,1,0,-1)	76 ± 5

Table 6.15. Series M results. Culture treatments are shown as symbols (defined in Table 6.13) representing the normalized variable concentrations (x_1, x_2, x_3, x_4).

Sporophyll source: Wilson Cove, San Clemente Island.

Culturing period: 7/27 - 8/10/79.

Culture	Treatment	Mean length and 95% confidence interval, μ (n=40)
M1	(-1,-1,-1,-1)	56 ± 4
M2	(-1,0,1,1)	91 ± 5
M3	(-1,1,0,0)	101 ± 5
M4	(0,-1,1,1)	64 ± 5
M5*	(0,0,0,0)	136 ± 6
M6	(0,1,-1,-1)	91 ± 6
M7	(1,-1,0,0)	87 ± 6
M8	(1,0,-1,-1)	103 ± 4
M9	(1,1,1,1)	50 ± 5

*M5 was the only culture to produce embryonic sporophytes within 14 days after spore release among the 27 treatments of Series L, M and N.

Table 6.16. Series N results. Culture treatments are shown as symbols (defined in Table 6.13) representing the normalized variable concentrations (x_1, x_2, x_3, x_4).

Sporophyll source: Cameo Shores, Corona del Mar.

Cutluring period: 11/13 - 11/27/79.

Culture	Treatment	Mean length and 95% confidence interval, μ (n=40)
N1	(-1,-1,0,1)	75 $\pm$ 5
N2	(-1,0,-1,0)	112 $\pm$ 6
N3	(-1,1,1,-1)	52 $\pm$ 5
N4	(0,-1,-1,0)	100 $\pm$ 7
N5	(0,0,1,-1)	94 $\pm$ 5
N6	(0,1,0,1)	105 $\pm$ 6
N7	(1,-1,1,-1)	24 $\pm$ 3
N8	(1,0,0,1)	110 $\pm$ 5
N9	(1,1,-1,0)	110 $\pm$ 6

Table 6.17. Series L, M, N Analysis of Variance.

Variance due to	df	SS	MS	F Ratio
Main Effects				
x_2	1	44,935	44,935	140*
x_3	1	132,356	123,356	412*
x_4	1	26,864	26,864	83.7*
Quadratic Effects				
x_1^2	1	64,027	64,027	195*
x_2^2	1	196,082	196,082	611*
x_3^2	1	42,507	42,507	132*
x_4^2	1	77,832	77,832	242*
First Order Interactions				
x_1x_3	1	22,688	22,688	70.7*
Higher Order Interactions				
$x_3x_1^2$	1	5,018	5,018	15.6*
$x_3x_2^2$	1	6,076	6,076	18.9*
Within subgroup	1053	341,605		
Lack of fit	16	1,492		
Error	1069	343,097	321	
Total	1079	961,482		

*Effect significant at 99.9% confidence level ($F_{0.999(1,1069)} = 11.2$).

Table 6.18. Optimal micronutrient levels based on Series L, M and N results*.

Model: $Y = 136 + 8x_2 - 5x_3 + 7x_4 - 7x_1x_3 - 15x_1^2 - 27x_2^2 - 12x_3^2 - 18x_4^2 - 6x_3x_1^2 - 6x_3x_2^2$,

where: Y = mean gametophytic length in microns,

x_1 = normalized Zn_T (as previously defined),

x_2 = normalized Mn_T,

x_3 = normalized Cu_T,

x_4 = normalized Co_T.

Variable	Normalized level	Estimated total concentration (nM)
x_1	0.06	169
x_2	0.2	28
x_3	-0.2	12
x_4	0.2	65

*Predicted maximum length = 138 microns.

Table 6.19. Comparison of observed mean gametophytic lengths (μ) for Series L with lengths (referred to as predicted lengths) determined by the LSQENP-generated equation (Table 6.18).

Culture	Observed mean length	Predicted length	%Error
L1	58	64	10
L2	96	96	0
L3	85	89	5
L4	77	76	-1
L5	118	118	0
L6	92	94	2
L7	86	87	1
L8	91	91	0
L9	76	77	1

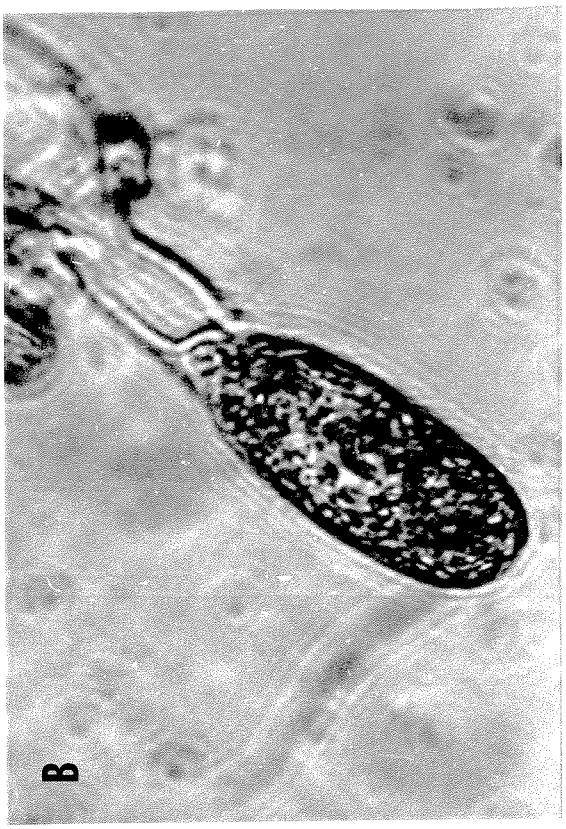
Table 6.20. Comparison of observed mean gametophytic lengths (μ) for Series M with lengths (referred to as predicted lengths) determined by the LSQENP-generated equation (Table 6.18).

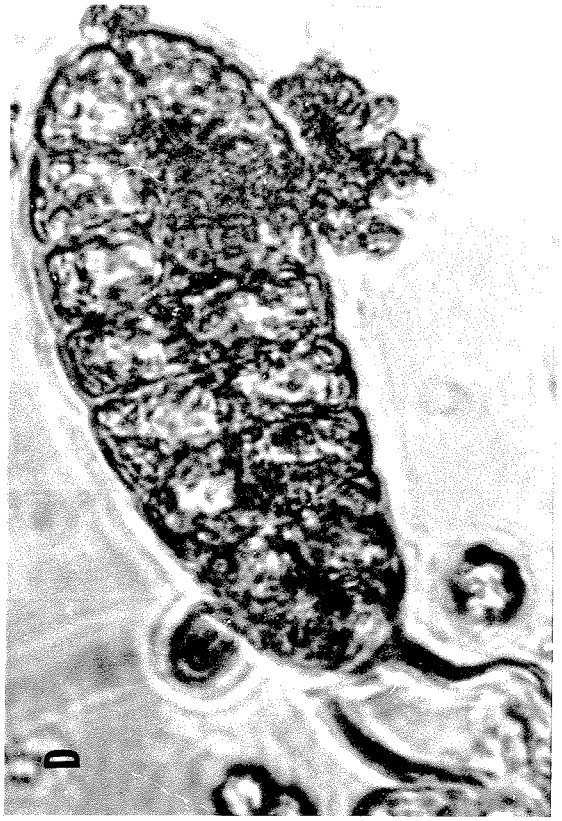
Culture	Observed mean length	Predicted length	%Error
M1	56	59	5
M2	91	94	3
M3	101	102	1
M4	64	67	5
M5	136	136	0
M6	91	91	0
M7	86	87	-1
M8	103	102	-1
M9	50	55	10

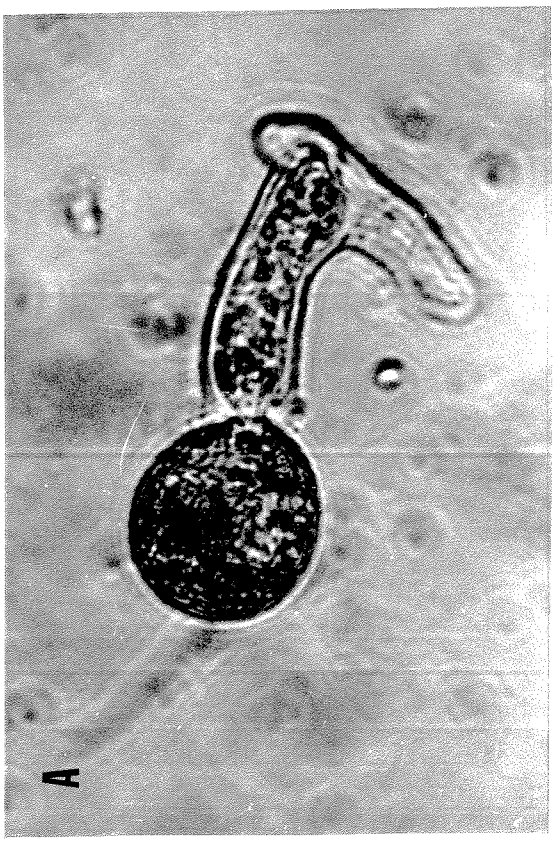
Table 6.21. Comparison of observed mean gametophytic lengths (μ) for Series N with lengths (referred to as predicted lengths) determined by the LSQENP-generated equation (Table 6.18).

Culture	Observed mean length	Predicted length	% Error
N1	71	75	6
N2	112	113	1
N3	52	55	6
N4	100	100	0
N5	94	94	0
N6	105	106	1
N7	24	25	1
N8	110	110	0
N9	110	114	4

Figure 6.4. Embryonic stages of Macrocystis pyrifera from culture M5. A. Release of oogonium from egg case (day 12). B. Single-celled sporophyte (day 12). C. Longitudinal cell reproduction in fixed and stained embryonic sporophyte (day 14). D. Transverse cell reproduction begins (day 14). Magnification = 400x.


B


D


A


C

treatments instead of 81). The price paid for this reduction was the confounding of certain effects called "aliases" (see Table 6.12). Two assumptions were made in selecting this design: 1) three-way and higher interactions were not significant, and 2) two-way linear interactions with x_4 (Co_T) were not significant. Results from Series L, M and N indicated, however, that the x_1x_3 (Zn-Cu) interaction, confounded with the x_2x_4 (Mn-Co) interaction, was significant. A 3^2 factorial experiment in Series P examined primary and interactive effects of manganese and cobalt to isolate this confounded effect (Figure 6.5). Combinations of each of three Mn_T and Co_T concentrations were used (Table 6.22). Aquil nutrient variables other than manganese and cobalt were held constant (Table 6.23). Media temperatures was set at 14^oC.

Series P results (Table 6.24) were fit to the equation:

$$Y = \sum_{j=0}^{2} \sum_{i=0}^{2} B_{ij} x_1^i x_2^j$$

where Y equals gametophytic length in microns, and x_1 and x_2 are normalized Mn_T and Co_T concentrations respectively (Table 6.25). Caltech Computing Center Library subroutine LSQENP was used to determine least squares estimates for coefficients B_{ij}. The analysis of variance indicated that the Mn-Co interaction was not significant at a 95% confidence level. The effect observed in Series L, M and N was therefore a Zn-Cu interaction. Series L, M and N also

Figure 6.5. Series P, a 3^2 factorial experiment where x_1 and x_2 are normalized Mn_T and Co_T concentrations respectively. Design points are formatted such that a(b,c) denotes culture number a at x_1, x_2 normalized levels c, d respectively.

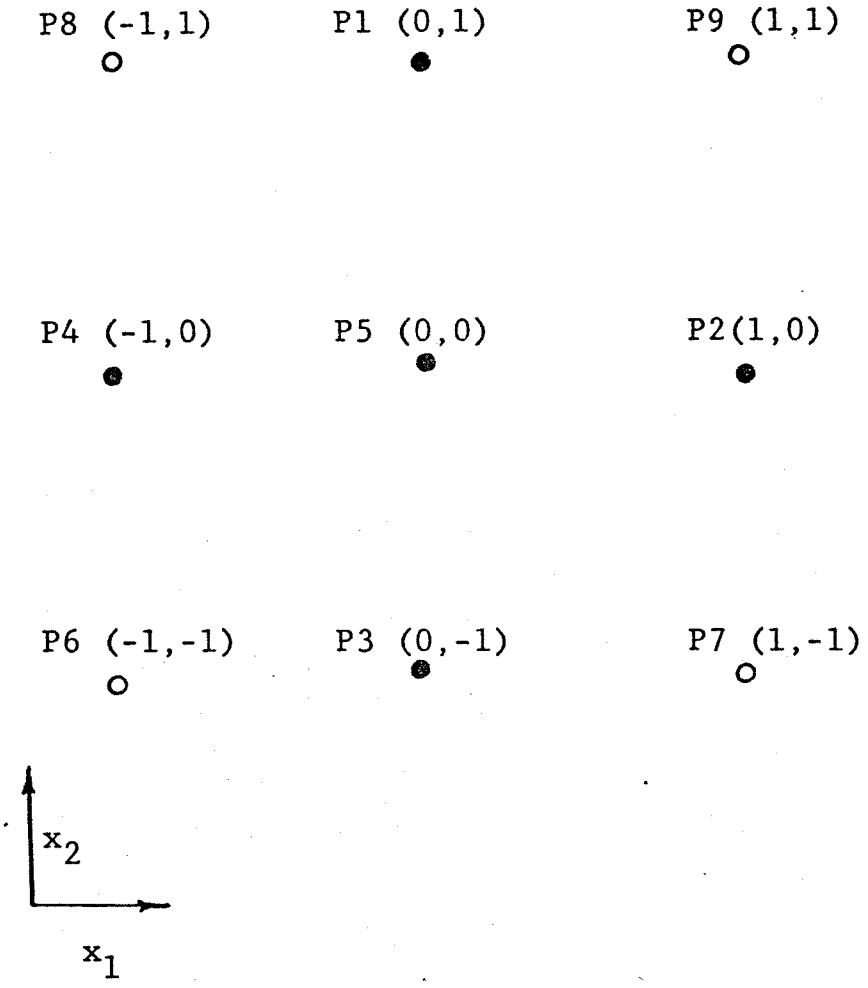


Table 6.22. Definition of symbols representing the normalized variable concentrations for Series P. Concentrations given in p units in the following format, (analytical concentration, computed free-ion concentration.

Variable	Symbols -1	0	+1
x_1 (Mn_T conc.)	(8.3,9.3)	(7.6,8.6)	(7.3,8.3)
x_2 (Co_T conc.)	(8.3,11.3)	(7.3,10.3)	(7.0,10.0)

Table 6.23. Aquil nutrients held constant in Series P.

Nutrient	Analytical concentration, (nM)	Computed free-ion concentration, (nM)	Major species (%)
Metals			
Fe^{3+}	350	$7x10^{-11}$	FeEDTA (100)
Cu^{2+}	10	$3x10^{-5}$	CuEDTA (99)
Zn^{2+}	170	0.09	ZnEDTA (100)
Ligands			
MoO_4^{2-}	100	100	Free ion (100)
$EDTA^{2-}$	$6x10^{3}$	$7x10^{-5}$	CaEDTA (89)
			FeEDTA (6)
NO_3^-	$2x10^{4}$	$2x10^{4}$	Free ion (100)
PO_4^{3-}	$2x10^{3}$	0.3	HPO_4^{2-} (51)
			$MgHPO_4$ (47)
I^-	100	100	Free ion (100)

Table 6.24. Series P results. Culture treatments are shown as symbols (defined in Table 6.22) representing the normalized variable concentrations (x_1, x_2).

Sporophyll source: Reef Point, Laguna Beach.

Culturing period: 2/25 - 3/10/80.

Culture	Treatment	Mean length and 95% confidence interval, u (n=40)
P1	(0,1)	123 ± 5
P2	(1,0)	114 ± 6
P3	(0,-1)	115 ± 5
P4	(-1,0)	100 ± 6
P5	(0,0)	145 ± 5
P6	(-1,-1)	73 ± 4
P7	(1,-1)	84 ± 5
P8	(-1,1)	82 ± 6
P9	(1,1)	96 ± 5

Table 6.25. Series P analysis of variance.

Model: $Y = 145 + 6x_1 + 5x_2 - 37x_1^2 - 25\ x_2^2$

where: Y = mean gametophytic length in microns,

x_1= normalized Mn_T (as defined in Table 6.22), and

x_2= normalized Co_T.

Variance due to	df	SS	MS	F ratio
Main effects				
x_1	1	11426	11426	41*
x_2	1	5881	5881	21*
Quadratic effects				
x_1^2	1	104835	104835	376*
x_2^2	1	49203	49203	176*
Within subgroup	351	98763		
Lack of fit	4	286		
Error	355	99049	279	
Total	359	270394		

*Effect significant at 99.9% confidence level ($F_{0.999(1,355)} = 11.3$)

indicated an unconfounded Zn-Cu interaction ($x_3x_1^2$) but no unconfounded cobalt interactions.

Optimal concentrations for Mn_T and Co_T of 27 and 60 n$\underline{M}$ respectively were indicated by Series P results (Table 6.26). Predicted and observed mean gametophytic lengths compared favorably (Table 6.27).

Ability of the Series L, M and N model to predict Series P results was examined (Table 6.28). Variation between predicted and observed mean gametophytic lengths ranged from 2 to 13%. Variations were greatest at the experimental design corner points (P6 to P9), because Series P results demonstrated even stronger manganese and cobalt quadratic effects than observed from Series L, M and N.

Table 6.26. Optimal manganese and cobalt total concentrations based on Series P results*.

Variable	Normalized level	Estimated total concentration (nM)
$x_1(Mn_T)$	0.08	27
$x_2(Co_T)$	0.1	60

Table 6.27. Comparison of observed mean gametophytic lengths (μ) for Series P with lengths (referred to as predicted lengths) determined by the LSQENP-generated equation (Table 6.25).

Culture	Observed mean length	Predicted length	%Error
P1	123	125	2
P2	114	114	0
P3	115	115	0
P4	100	102	0
P5	145	145	0
P6	73	72	-1
P7	84	84	0
P8	82	82	0
P9	96	94	-2

*Predicted maximum length = 145 microns.

Table 6.28. Comparison of observed mean gametophytic lengths for Series P and lengths predicted for Series P cultures from the Series L, M and N model. Culture treatments given as normalized variable levels (Zn_T, Mn_T, Cu_T, Co_T). Zn_T and Cu_T were fixed at 170 nM and 10 nM respectively.

Culture	Treatment	Observed mean length (μ)	Predicted length	% Error
P1	(0.06,0,-0.33,1)	123	125	2
P2	(0.06,1,-0.33,0)	114	119	4
P3	(0.06,0,-0.33,-1)	115	111	-3
P4	(0.06,-1,-0.33,0)	100	103	3
P5	(0.06,0,-0.33,0)	145	138	-5
P6	(0.06,-1,-0.33,-1)	73	78	7
P7	(0.06,1,-0.33,-1)	84	94	12
P8	(0.06,-1,-0.33,1)	82	92	12
P9	(0.06,1,-0,33,1)	96	108	13

CHAPTER 7

DISCUSSION

Algal Culturing Media.

Significant differences in formulation and micronutrient speciation exist between the final Aquil medium and those of other mariculturing media (Tables 7.1 and 7.2). These differences would almost certainly affect gametophytic performances substantially if only because required elements are lacking. Examples are found in the media described by Taylor (1963).

Optimal total concentrations in Aquil for the five metallic micronutrients, Co, Cu, Fe, Mn, and Zn were consistently higher than concentrations found in typical coastal seawater (Table 7.1). Using micronutrient concentrations specified by Brewer (1975) and assuming 20% copper-organic ligand complexation (Lieberman, unpublished), seawater speciation was computed (Table 7.2). Although total micronutrient concentrations were higher for Aquil than for coastal seawater, computed free-ion concentrations were comparable or even lower in Aquil (e.g., zinc and copper). Assuming metallic micronutrients in uncomplexed form are more readily available to the plant (Sunda and Guillard, 1976), higher total metal concentrations in Aquil relative to natural seawater may have been required to overcome metal-EDTA complexation in Aquil. EDTA was added to Aquil to prevent ferric hydroxide precipitate. An additional consequence of EDTA addition was that

Table 7.1. A Comparison of Constituents Among a Variety of Mariculture Media.*

Element	Synthetic Media			Coastal Seawater	Aquil Formulations	
	ASP - 2	B-1	CF-1		Initial unsuccessful	Final successful
			Major Elements, mM			
Na	314.4	316.4	310,0	468	480,0	480.0
Mg	20.3	20,3	20,3	53.2	54,6	54,6
K	8.1	8,1	8,2	10,2	9,4	9,4
Ca	2,5	0,9	0,68	10,2	10.5	10,5
Cl	318,6	317,0	316,8	546.0	559,0	559,0
S	20,3	20,3	20,3	28,2	28,8	28,8
C	-	2,4	2,4	2,3	2,4	2,4
N**	5,9	5,9	2,0	0,04	0,03	0,02
P	0,03	0,02	0,10	0,002	0,002	0,002
B	0,55	0,58	-	0,41	0,50	0,50
Si	0,53	0,18	0,10	0,07	-	-
Br	-	-	-	0,84	0,80	0,80
F	-	-	-	0,07	0,07	0,07
Sr	-	-	-	0,09	0,06	0,06

Table 7.1 (continued)

Trace Elements, μM

Element	Synthetic Media ASP-2	B-1	CF-1	Coastal Seawater	Aquil Formulations Initial unsuccessful	Final successful
Fe	14.3	5.0	5.2	0.035	0.10	0.35
Mn	21.8	21.8	10.0	0.0036	0.01	0.03
Co	0.05	0.5	0.5	0.0008	0.01	0.07
Zn	2.3	2.3	1.6	0.076	0.10	0.17
Cu	0.02	-	-	0.008	0.01	0.01
Na_2EDTA	80.6	80.6	-	-	8.0	6.0
Mo	-	-	-	0.1	0.10	0.10
I	-	-	-	0.5	0.10	0.10
Initial pH	7.6-7.8	7.6-7.8	7.5-7.8	8.0	8.05-8.15	8.05-8.15
N/P	196:1	295:1	20:1	15.6:1	15:1	10:1
Mg/Ca	8.1	23:1	30:1	5.3:1	5.2:1	5.2:1

*ASP-2, B-1, CF-1 taken from Taylor (1963) (ASP-2 is from Provasoli (1957). B-1 is a modified Provasoli medium. CF-1 is Taylor;s chelate-free medium). Values for coastal seawater were from Brewer (1975).

**excluding N_2

Table 7.2. Comparison of micronutrient speciation among various mariculture media.

Nutrient	Medium[1]	Analytical concentration, nM	Computed free-ion concentration, nM	Major species (%)
Metals				
Fe^{3+}	a	350	$7x10^{-11}$	FeEDTA (100)
	b	35	$2x10^{-10}$	$Fe(OH)_3$(s) (97)
	c	22	$2x10^{-10}$	" (91)
	d	$5x10^3$	10^{-9}	" (100)
Mn^{2+}	a	30	3	MnEDTA (65)
	b	4	1	$MnCl^+$ (64)
	c	7	2	$MnCl^+$ (64)
	d	10^4	$4x10^3$	$MnCl^+$ (49)
Co^{2+}	a	70	0.07	CoEDTA (99)
	b	0.8	0.1	$CoCl^+$ (63)
	c	11	1	$CoCl^+$ (55)
	d	500	100	$CoCl^+$ (65)
Cu^{2+}	a	10	$4x10^{-5}$	CuEDTA (99)
	b	8	0.004	$Cu(BOH_4)_2$ (63)
	c	11	0.005	" (52)
	d	-[2]	-	-
Zn^{2+}	a	170	0.09	ZnEDTA (100)
	b	76	7	$ZnCl^+$ (61)
	c	15	1	$ZnCl^+$ (50)
	d	$2x10^3$	200	$ZnSiO_3$(s) (51)
Ligands				
MoO_4^{2-}	a	100	100	Free ion (100)
	b	0.1	0.1	" "
	c	N/A[3]	N/A	
	d	-	-	-
$EDTA^{2-}$	a	$6x10^3$	$7x10^{-5}$	CaEDTA (89)
	b[4]	30	10^{-7}	ZnEDTA (63)
	c	30	10^{-7}	CaEDTA (63)
	d	-	-	-

Table 7.2 (continued)

Nutrient	Medium	Analytical concentration, nM	Computed free-ion concentration, nM	Major species (%)
Ligands (continued)				
NO_3^-	a	$2x10^4$	$2x10^4$	Free ion (100)
	b	$4x10^4$	$4x10^4$	" "
	c	$2.5x10^4$	$2.5x10^4$	" "
	d	$2x10^6$	$2x10^6$	" "
PO_4^{3-}	a	$2x10^3$	0.3	HPO_4^{2-} (51)
	b	$2x10^3$	0.3	" "
	c	$2x10^3$	0.3	" "
	d	10^5	8	HPO_4^{2-} (75)
I^-	a	100	100	Free ion (100)
	b	0.1	0.1	" "
	c	N/A	N/A	
	d	-	-	-

[1]Type of medium denoted by lower case letters:
a = final Aquil formulation, pH = 8.1,
b = coastal seawater (Brewer, 1975), pH = 8.0
c = 300 m seawater (station 6 Km south of Newport Bay, 5/5/77), pH=8.0.
d = CF-1 medium (Taylor, 1963), pH - 7.7.

[2]Symbol "-" denotes that the micronutrient was not included in the formulation.

[3]N/A denotes that analysis for that micronutrient was not performed.

[4]Organics in media b and c were estimated by the concentration of EDTA required to produce 20% copper-organic ligand complexation (Lieberman, unpublished).

all five metallic micronutrients in Aquil (Co, Cu, Mn and Zn in addition to Fe) were primarily complexed by EDTA (Table 7.1). In contrast, the NO_3-N concentration in the final Aquil formulation was within the normal range found in seawater since NO_3-N exists almost entirely as the uncomplexed form in both seawater and Aquil.

Implications for Fertilizing with Deep Seawater.

Why did Macrocystis gametophytes cultured in Monterey Bay surface water (MBSW) exhibit rapid growth and development by comparison with gametophytes cultured in a 1:1 mixture of surface and deep (300 m) seawater (KMLSW) obtained 6 km south of Newport Bay (see Chapter 5, Preliminary Experiments)? Phytoplankton growth stimulation during natural upwelling periods has been correlated with increased macronutrient concentrations in surface water (Corredor, 1979). Influences from macronutrients cannot, however, explain growth rate differences obtained for MBSW and KMLSW cultures (see Table 5.1).

Certain plants can break Fe-chelator and/or Fe-inorganic ligand bonds, thereby rendering these complexes biologically exploitable (Romheld and Marschner, 1979). If Macrocystis can readily use complexed forms of Co, Cu, Fe, Mn and Zn, it can be inferred that unenriched MBSW was deficient in micronutrients for optimal gametophytic growth (Table 7.3). Experimental evidence (see Chapter 5, Preliminary Experiments) obviously contradicts this hypothesis.

If micronutrients in free ionic form are accessible to Macrocystis, then only manganese occurred in MBSW at a

Table 7.3. Concentration ratios* relating Monterey Bay surface water (MBSW) to Aquil.

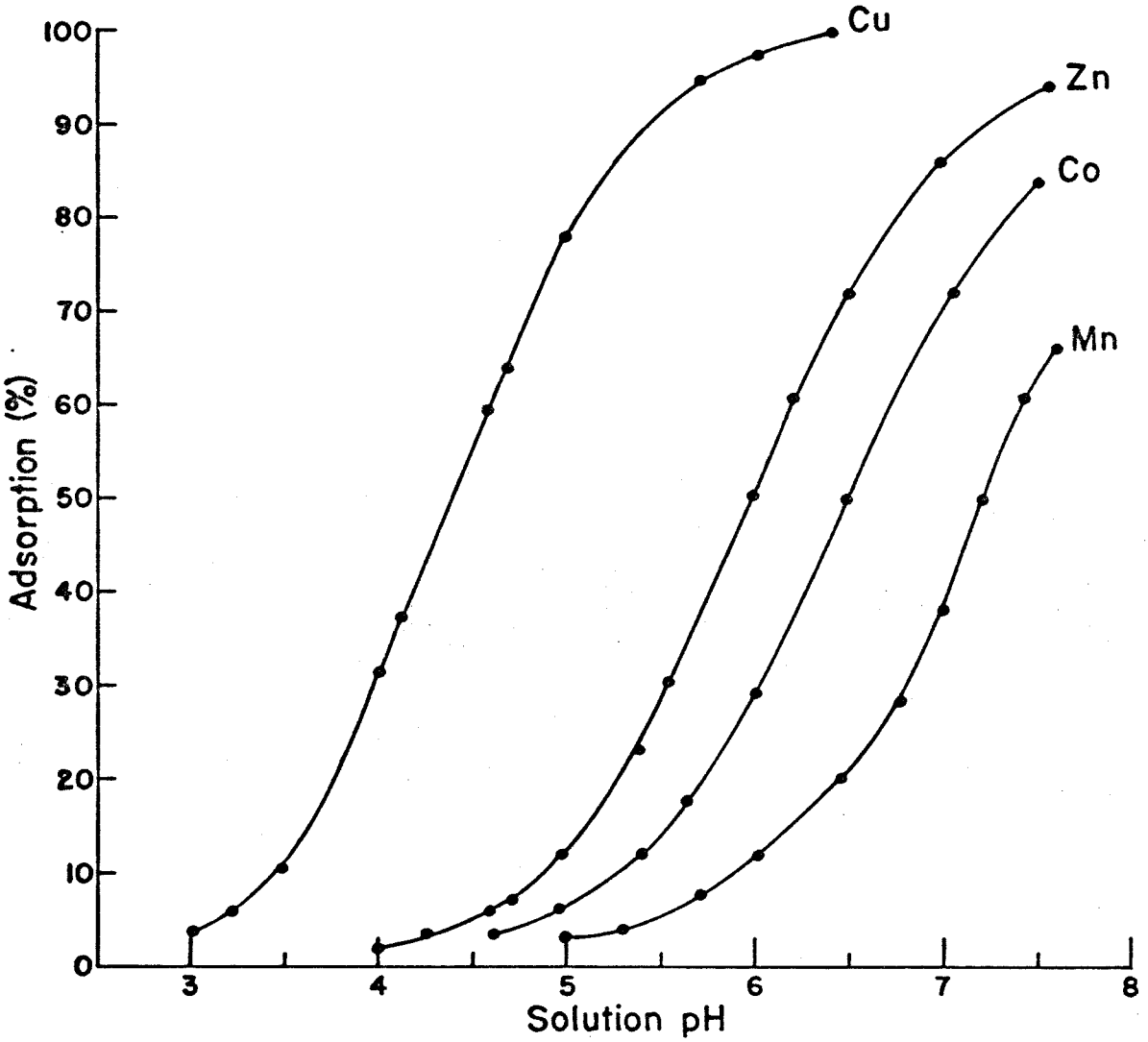
Nutrient	Concentration ratio based on analytical concentration	Concentration ratio based on computed free-ion concentration
Co(II)	4×10^{-1}	6×10^{1}
Cu(II)	2	3×10^{2}
Fe(III)	2×10^{-1}	3
Mn(II)	7×10^{-1}	2
Zn(II)	4×10^{-1}	7×10^{1}

*Concentration ratio = $\frac{[Me]_{MBSW}}{[Me]_{final\ Aquil}}$ using concentrations from Tables 5.1 and 7.2 ([Me] refers to the concentration of a tabled metallic nutrient.)

concentration suited for gametophytic culturing. High concentrations in MBSW for Cu, Zn and Co indicated that plants cultured in this medium should have been strongly inhibited. MBSW/Aquil ratios for Cu, Zn and Co were 200, 67 and 43 respectively based on computed free-ion concentrations (Table 7.3). Amorphous ferric hydroxide in seawater (Table 7.2), however, provides adsorption sites which may remove trace elements from solution. Grimme (1968) investigated Mn(II), Co(II), Cu(II) and Zn(II) adsorption onto goethite (α-FeOOH) at 10^{-5} M initial Me(II) concentration (Figure 7.1). If adsorption curves are shifted one pH unit to the right to account for seawater ionic strength (Vuceta, 1976) nearly all Cu(II) is adsorbed at pH = 8. Zn(II) is adsorbed to a lesser degree, followed by Co(II) and finally Mn(II). Adsorption percentages of 98.3%, 99.7%, 50.0% and 98.6% were required to reduce the free-ion concentration ratios of Table 7.3 to 1 for Co, Cu, Mn and Zn respectively.

The averages of surface and 300 m seawater trace metal concentrations (see Table 5.1) sampled 18 September to 3 November 1976 were 1.5 nM Co_T, 31 nM Cu_T, 1.5 nM Mn_T and 34 nM Zn_T. Using adsorption percentages estimated for MBSW, the KMLSW:Aquil free-ion concentration ratios for Co, Cu, Mn and Zn were 0.04, 2, 0.07, and 0.5 respectively. Thus cobalt and manganese were apparently greatly deficient in KMLSW.

Figure 7.1. Adsorption of Cu, Zn, Co, and Mn onto goethite as a function of solution pH (Grimme, 1968) with $Me(II)_T = 10^{-5}\underline{M}$, $S = 48.5 \pm 0.2\ m^2/\ell$ goethite (Balistrieri, 1977).



Trace element analysis of KMLSW and MBSW indicated total iron concentrations of 18 nM and 72 nM respectively (Table 5.1). Removal of 67 nmoles combined of Co(II), Cu(II), Mn(II) and Zn(II) from solution per liter KMLSW was required to decrease free ion concentration ratios to the same degree as MBSW. Using adsorption site data for amorphous ferric hydroxide (Davis and Leckie, 1978), iron in KMLSW could have adsorbed only 16 nmoles of trace elements per liter (i.e., leaving removal of 51 nM trace nutrients that cannot be attributed to adsorption onto ferric hydroxide). With insufficient iron in KMLSW to remove trace nutrients from solution one might question the cobalt and manganese deficiency proposed above. Similar calculations using MBSW trace element data indicate, however, that only 63 nmoles of trace nutrients could have been adsorbed onto ferric hydroxide in MBSW (removal of a total of 116 nmoles of other trace elements per liter was required), leaving unaccounted 53 nmoles per liter. The culturing success achieved using MBSW (see Chapter 5) and the nearly identical values for unaccounted trace nutrient removal in KMLSW and MBSW (51 and 53 nM respectively) suggest that another process(es) (e.g., adsorption onto organic or siliceous particulates) was responsible for additional trace removal from these media.

Summary.

A formulation for the chemically defined medium, Aquil, was derived that sustained gametogenesis and yielded multicellular embryonic sporophytes of Macrocystis within a two-week culturing period. Multicellular sporophytes have been consistently produced ever since Series J cultures (4/23/79). Gametophytic growth was similar regardless of the source of sporophylls used for initiating a culture (Series J, K and M).

Nine nutrients (Co, Cu, Fe, I, Mn,Mo, N, P, Zn) were required for completion of the life cycle by Macrocystis. All must be considered essential elements for continuing existence of this commercially and ecologically important brown alga.

Optimal total concentrations in Aquil were determined for five micronutrients (Co, Cu, Fe, Mn, Zn) and NO_3-N. Primary and interactive effects were examined by varying micronutrient concentrations in the general region of the optimal level. All micronutrients exhibited highly significant linear and/or quadratic main effects (only the guadratic main effect was significant for zinc). Iron-zinc, copper-manganese and copper-zinc interactions were also significant at the 99.9% confidence level. Main and interactive inhibition of Macrocystis gametophytes by copper demonstrated the sensitivity to nanomolar fluctuations of this element.

This study represents a step toward understanding micronutrient effects on marine algae. Innumerable culturing problems may be examined by means of chemically defined media. Separate studies are underway to compare micronutrient requirements reported here for gametophytes with those of juvenile Macrocystis sporophytes, one or more cm in length. Results from Series I suggest that optimal concentrations for nitrate may differ between gametophytes and juvenile sporophytes.

Main and interactive effects of other Aquil nutrients should be examined (e.g. I, Mo, P). Relationship between molybdenum and nitrogen assimilation in Macrocystis could be investigated using Aquil. Recent unpublished work by Manley at KML on Macrocystis juvenile sporophytes indicated a half-saturation constant (K_m) of 10^{-5}M for iodide. This was two orders of magnitude higher than the iodide concentration employed for gametophyte culturing experiments discussed in this thesis. Correlations existed between N:P ratios in deep seawater and in phytoplankton composition (Richards, 1965). Effects on Macrocystis gametophytic growth and composition from varying N:P ratios in Aquil could be compared to effects observed on Macrocystis juvenile sporophytes in enriched seawater cultures (North, 1978b). Effects on adult Macrocystis growth rates from seasonal changes in nutrient seawater speciation are being examined at KML.

Selectivity of algae for various micronutrient species could be examined with a chemically defined medium (e.g. by

using chelating agents other than EDTA for selectively binding micronutrients of interest).

APPENDIX I

AQUIL PREPARATION MANUAL

The following manual was written for use in the Kerckhoff Marine Laboratory Cleanroom. It serves as a guide for those using the modified Aquil system. Little or no laboratory experience is assumed. Sections X (the original Aquil reference [Morel et al., 1975]) and XI (a compilation of equipment manuals and brochures) are not included in this appendix.

Appendix I (continued)

TABLE OF CONTENTS

Appendix I (continued)

General Procedure.

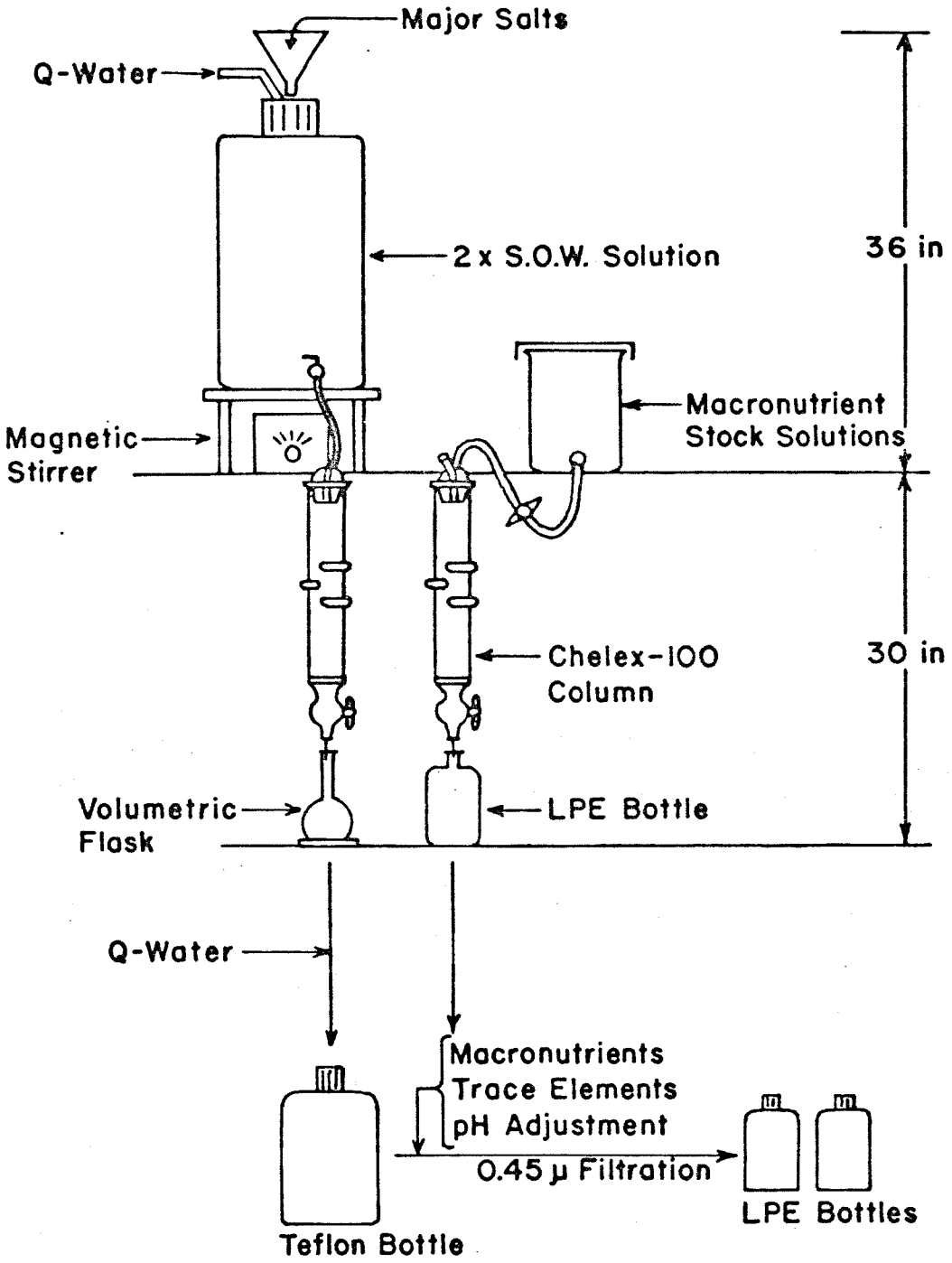
Aquil is prepared in the Kerckhoff Marine Laboratory Cleanroom. The schematic diagram (Figure A) should help in understanding directions below.

1. Elute the first 300 mL of Major Salts Solution (2x Standard Ocean Water (S.O.W.)) through a Chelex-100 column into a beaker and discard.
2. Elute 200 mL of Major Salts Solution into a 1-liter volumetric flask. Swirl and drain flask. Elute another liter into the flask (never run columns dry.)
3. Pour contents of flask into a 2-liter Teflon bottle. Add one liter Q water to the bottle.
4. Add trace metals and macronutrients according to desired concentrations (refer to sections on spiking volumes.)
5. Adjust pH to 8.1 and record (refer to section on pH Adjustment.)
6. Filter 300 mL of Q water through the millipore filtering unit (Figure B). Filter 100 mL from the 2-liter Teflon bottle to condition the filter. Remove the waste bottle and replace with a 1-liter Aquil storage bottle.
7. Filter the remaining 1900 mL of Aquil into two storage bottles. Cover and refrigerate the final

product. Do not store over two weeks.

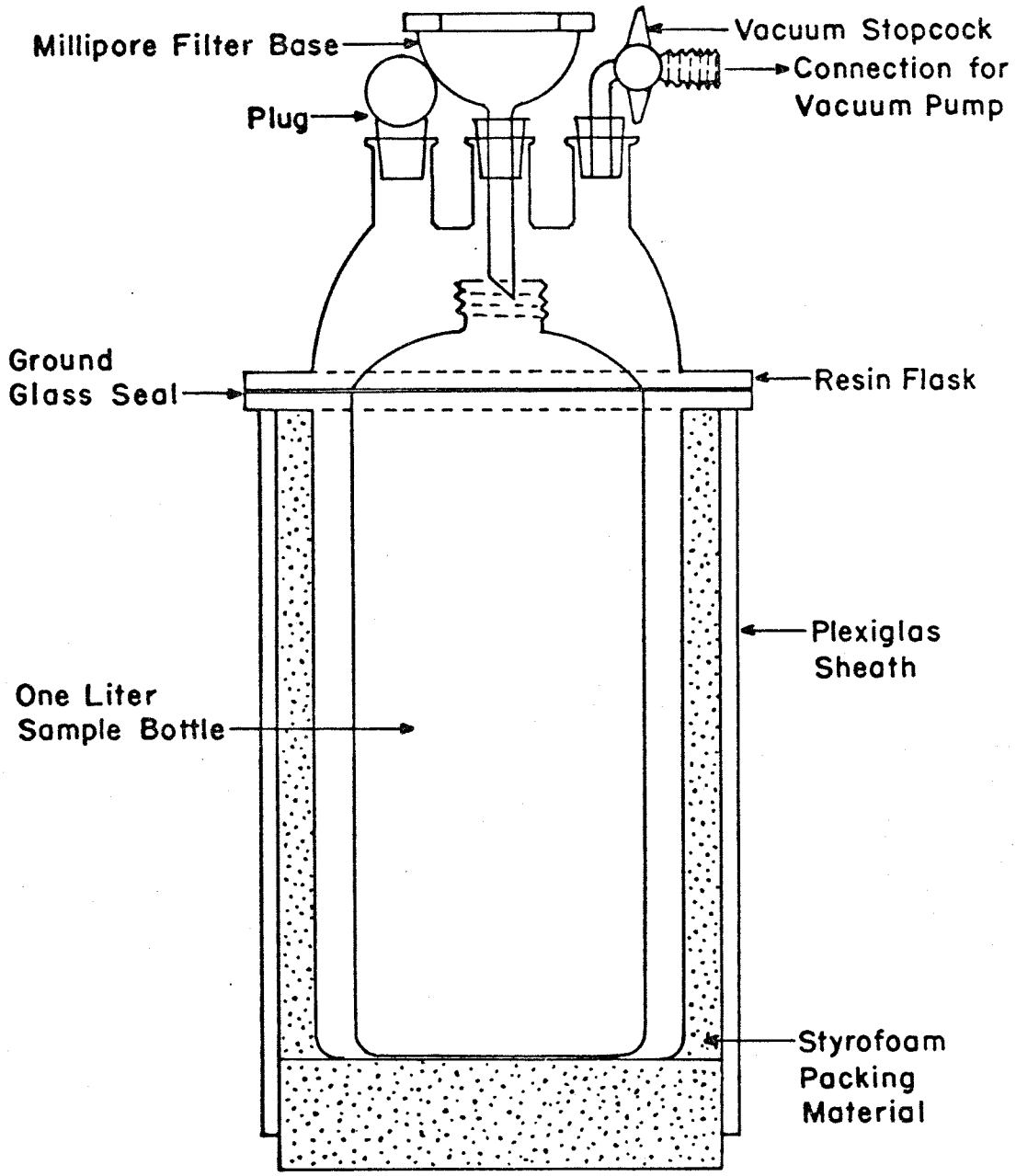
This completes the production procedure. The original paper by Morel et al.(1975), cited in section X of this manual, may be examined for further detail. Procedures for cleaning glassware and plasticware are described in the following section.

Figure A. Aquil production system, Kerckhoff Marine Laboratory Cleanroom.



Production Time: 1 hr/2-

Figure B. Millipore Filtering Unit



Appendix I (continued)

General Cleaning Procedure.

All glassware and plasticware that are used to produce Aquil must be acid washed. Acid washing denotes the following standard operating procedure.

1. rinse the item in 50% HNO_3,
2. soak the item in 50% HNO_3 for at least three days,
3. drain and rinse again in 50% HNO_3,
4. rinse twice with Q water,
5. soak in Q water for at least one day,
6. drain and rinse again in Q water,
7. if the item is not immediately used, wrap it in Saran wrap.

The reason for employing Aquil is that it provides a trace element defined culturing medium. Therefore, one should be especially careful to avoid inadvertent contamination of media (e.g., prolonged exposure to atmosphere). Aquil preparation is not difficult, but these procedures should be followed meticulously.

Appendix I (continued)

Initial Cleaning of Linear Polyethylene (LPE).

One source of culture contamination is the leaching of trace metals from linear polyethylene preparation and storage bottles (Moody and Lindstrom, 1977). The following standard operating procedure is recommended for initial cleaning of LPE equipment. Following the completion of this procedure on a new LPE item, the General Cleaning Procedure described above may be used.

1. rinse once in 50% HCl,
2. soak one week in 50% HCl,
3. rinse twice with Q water,
4. rinse once with 50% HNO_3,
5. soak one week in 50% HNO_3,
6. rinse twice with Q water,
7. fill and soak at least one week in Q water before use.

Appendix I (continued)

Chelex-100 Column Efficiency.

After 10 liters of solution have been eluted through the Chelex-100 column, results of AAS analyses done on unspiked Aquil indicated significant trace element contamination due to reduced column efficiency. This is particularly true for copper and zinc.

High cost of Chelex-100 and large volumes of Aquil required for culture studies may dictate that the resin be reused. Therefore, after 5 liters of solution have been eluted through the column, the Chelex-100 may be regenerated as the H^+ form as follows:

1. transfer resin into a beaker,
2. adjust pH to 2.0 or lower and let set for an hour,
3. rinse twice with Q water,
4. adjust pH to 8.5,
5. rinse twice with Q water and repack the column making sure that no air bubbles form.

Appendix I (continued)

pH Adjustment.

Chemical speciation in Aquil is pH dependent (Table A). Aquil pH is adjusted to 8.1 in step 3 of the General Procedure. This adjustment procedure is outline below. After thoroughly mixing the solution from step 2 of the General Procedure:

1. pour 5 mL from the 2-liter Teflon storage bottle into a beaker,
2. pipette 2 mL from the beaker into an LPE vial containing a Teflon microstirrer,
3. adjust pH in the vial to 8.10 $\pm$ 0.05* with dilute spectrograde Na_2CO_3 and HCl. Record the total volume added.
4. add proportional volumes of concentrated base or acid to the 2-liter Teflon storage bottle and stir for at least two minutes,
5. rinse beaker and vial with Q water,
6. again, pour about 5 mL from the 2-liter bottle into the beaker. Pipette out 2 mL into the vial and check the pH. If the pH is not within 0.05 units of 8.10, repeat steps 3 through 6**,
7. record final pH using the printer (Orion Research Inc., Cambridge, MA, Model 751) and attach the recorded printout to the Aquil notebook or save for your records.

TABLE A. SPECIATION OF SOME TRACE METALS AND LIGANDS IN AQUIL (Effect of pH)
An Equilibrium Computation using the REDEQL2 Program.

	Analytical Conc. (M)	Computed Free Ion Activity (M) pH=8.0	pH=8.1	pH=8.2	Major Species pH=8.0		pH=8.1		pH=8.2	
Iron	1.8×10^{-8}	1.6×10^{-19}	7.9×10^{-20}	4.0×10^{-20}	FeEDTA	29%	FeEDTA	19%	FeEDTA	12%
					$Fe(OH)_3(s)$	71%	$Fe(OH)_3(s)$	81%	$Fe(OH)_3(s)$	88%
Manganese	3.2×10^{-8}	9.2×10^{-9}	9.2×10^{-9}	9.0×10^{-9}	$MnCl^+$	60%	$MnCl^+$	60%	$MnCl^+$	61%
					Free	29%	Free	29%	Free	29%
Cobalt	3.2×10^{-8}	8.4×10^{-10}	8.0×10^{-10}	7.8×10^{-10}	$CoEDTA^-$	83%	$CoEDTA^-$	84%	$CoEDTA^-$	84%
					$CoCl^+$	12%	$CoCl^+$	12%	$CoCl^+$	12%
Copper	1.6×10^{-8}	1.4×10^{-12}	1.3×10^{-12}	1.4×10^{-12}	CuEDTA	88%	CuEDTA	84%	CuEDTA	97%
					$CuBOH_4$	10%	$CuBOH_4$	13%	$CuBOH_4$	2%
Zinc	6.3×10^{-8}	9.0×10^{-10}	8.5×10^{-10}	8.2×10^{-10}	$ZnEDTA^-$	89%	$ZnEDTA^-$	89%	$ZnEDTA^-$	89%
					$ZnCl^+$	9%	$ZnCl^+$	9%	$ZnCl^+$	9%
Molybdate	1.5×10^{-9}	1.5×10^{-9}	1.5×10^{-9}	1.5×10^{-9}	Free	100%	Free	100%	Free	100%
Arsenate	1.0×10^{-8}	1.2×10^{-11}	1.5×10^{-11}	1.9×10^{-11}	$HAsO_4^=$	100%	$HAsO_4^=$	100%	$HAsO_4^=$	100%
EDTA	3.2×10^{-7}	2.3×10^{-15}	2.5×10^{-15}	2.5×10^{-15}	CaEDTA	62%	CaEDTA	64%	CaEDTA	65%
					ZnEDTA	18%	ZnEDTA	18%	ZnEDTA	18%
Nitrate	1.0×10^{-5}	1.0×10^{-5}	1.0×10^{-5}	1.0×10^{-5}	Free	100%	Free	100%	Free	100%
Selenite	1.0×10^{-8}	5.4×10^{-9}	6.0×10^{-9}	6.5×10^{-9}	Free	54%	Free	60%	Free	65%
					$HSeO_3^-$	46%	$HSeO_3^-$	40%	$HSeO_3^-$	35%

I = 0.7 M

$-\log(pCO_2) = 3.5$

Appendix I (continued)

* pH measured by the electrode is dependent on ionic strength and temperature of the sample. The pH value of 8.1 assumes:

1. the ionic strength set by the Aquil major salts, and
2. a 15°C difference between preparation and culturing temperatures. pH increases by approximately 0.009 units per degree centigrade decrease.

If these assumptions are not satisfied, refer to Appendices IA and IB for corrections.

**The combination pH electrode should be thoroughly rinsed with Q water before it is positioned in the vial. The electrode is stored in pH=4 buffer. Failure to properly rinse the electrode before use will result in enormous errors for the adjustment volumes in step 3 of the pH adjustment procedure.

Appendix I (continued)

Metallic Impurities Introduced by pH Adjustment.

Adjustment of Aquil pH (to 8.1) is accomplished with about 2 mL 0.5 M Na_2CO_3 (10.6 g Ultrex sodium carbonate/ 200 mL Q water).

$FW_{Na_2CO_3} = 105.99$

$g\text{-}Na_2CO_3$ added/ 1-Aquil = 106 g/2-1 x $2x10^{-3}$ 1/1-Aquil

= 0.106 $g\text{-}Na_2CO_3$ / 1-Aquil.

Element	Specified Impurity (ppm)	Impurity Added (nM)	Typical Spiking Level (nM)
Co	< 0.01	< 0.02	10
Cu	0.1	0.2	10
Fe	0.1	0.2	100
Mn	0.005	0.01	10
Zn	< 0.2	< 0.3	100

Appendix I (continued)

Macronutrient Stock Solutions.

The three Macronutrient Stock Solutions are prepared as follows:

1. Phosphorus Stock Solution. Dissolve 0.544 g KH_2PO_4 in a 2-liter volumetric flask and fill to the mark with Q water. Pass solution through the Chelex-100 column as described below (P_T = 2.000 mM).
2. Nitrate Stock Solution. Dissolve 3.400 g $NaNO_3$ in a 2-liter volumetric flask and fill to the mark with Q water. Pass solution through the Chelex-100 column as described below (N_T = 20.00 mM).
3. Ammonium Stock Solution. Dissolve 2.140 g NH_4Cl in a 1-liter volumetric flask and fill to the mark with Q water. Pass solution through the Chelx-100 column as described below (N_T = 20.00 nM).

Stock solutions should not be kept for over three months. Solutions should be kept refrigerated when not in use.

Elution Procedure for Macronutrient Stock Solutions.

1. Adjust pH of solution to 8.1 $\pm$ 0.1.
2. Elute 300 mL of stock solution into a container and discard this volume.
3. Elute 200 mL of stock solution into a 1-liter LPE

Appendix I (continued)

bottle, swirl and drain.

4. Elute 1 liter of stock solution into the 1-liter LPE bottle.
5. Label, date and wrap the bottle for storage.

Appendix I (continued)

Macronutrient Spiking Levels.

Unlike Morel's procedure (Morel et al., 1975) for preparing Aquil (cited in section X) there is no Mixed Nutrient Solution. Rather, macronutrient stock solutions are themselves eluted as described above. Therefore, using the solutions prepared as described above, macronutrients are individually added as follows:

Stock Solution	Add	For every
Phosphorus	1 mL	1 μM desired
Nitrate-N	1 mL	10 μM desired
Ammonium-N	1 mL	10 μM desired.

Addition of macronutrient and micronutrient stock solutions into the 2-liter Teflon storage bottle is done with an autopipette. Whenever pipetting stock solutions, do not pipette directly out of the storage LPE bottle. Rinse a 50 mL LPE bottle with stock solution. Pour the approximate volume desired from the storage bottle into the smaller bottle. Then pipette from the smaller bottle. It is especially important that this procedure is followed closely during trace element additions because the volumes added are orders of magnitude smaller. Once one becomes familiar with the use of the autopipette, consistent microliter additions of nutrient stock solutions should be routine.

Appendix I (continued)

Major Salts Solution (2xS.O.W.).

The Major Salts Solution is prepared in 25-liter batches in a polyethylene aspirator bottle. To prepare the solution, follow this procedure:

1. measure out the salts:
   a. 1226.5 g NaCl
   b. 555.0 g $MgCl_2 \cdot 6H_2O$
   c. 204.5 g Na_2SO_4
   d. 77.0 g $CaCl_2 \cdot 2H_2O$
   e. 35.0 g KCl
   f. 10.0 g $NaHCO_3$
   g. 5.0 g KBr
   h. 1.5 g H_3BO_3
   i. 0.85 g $SrCl_2 \cdot 6H_2O$
   j. 0.15 g NaF.
2. fill aspirator bottle to 20-liter mark,
3. slowly add the salts and allow to dissolve (use magnetic stirrer,)
4. fill to 25-liter mark.

The Major Salts Solution is now ready for elution through the Chelex-100 column.

Appendix I (continued)

Trace Element Stock Stolutions.

There are presently nine Trace Element Stock Solutions. This number may change as interests in certain metals and ligands change. Procedures for preparing the Trace Element Stock Solutions are given below. Refrigerate and plastic wrap all stock solution storage bottles.

1. Arsenic Stock Solution. Dissolve 0.312 g $Na_2HAsO_4 \cdot 7H_2O$ in a 100 mL volumetric flask, and fill to the mark with Q water. Pour about 20 mL into an LPE bottle. Cover the bottle and agitate for one minute. Pour contents out and pour the remaining 80 mL from the volumetric flask into the bottle (As_T = 0.010 M).

2. Cobalt Stock Solution. Dissolve 0.238 g $CoCl_2 \cdot 6H_2O$ in a 1-liter volumetric flask, and fill to the mark with Q water. Rinse a 250-mL LPE bottle with the stock solution, then fill the bottle (Co_T = 0.001 M).

3. Copper Stock Solution. Dissolve 0.170 g $CuCl_2 \cdot 2H_2O$ in a 1-liter volumetric flask, and fill to the mark with Q water. Rinse a 250-mL LPE bottle with the stock solution, then fill the bottle (Cu_T = 0.001 M).

4. Iodine Stock Solution. Dissolve 0.166 g KI in a 1-liter volumetric flask and fill to the mark with Q water. Rinse a 250-mL LPE bottle with the stock solution, then fill the bottle (I_T = 0.001 M).

Appendix I (continued)

5. Iron Stock Solution. Refer to FeEDTA Stock Solution.

6. Manganese Stock Solution. Dissolve 0.198 g $MnCl_2 \cdot 4H_2O$ into a 1-liter volumetric flask, and fill to the mark with Q water. Rinse a 250-mL LPE bottle with the stock solution then fill the bottle (Mn_T = 0.001 M).

7. Molybdenum Stock Solution. Dissolve 0.242 g $Na_2MoO_4 \cdot 2H_2O$ in a 100-mL volumetric flask and fill to the mark with Q water. Pour about 20 mL of the solution into a 250-mL LPE bottle; cover the bottle and shake vigorously for one minute. Pour out the 20 mL and pour the remaining 80 mL from the volumetric flask into the bottle (Mo_T = 0.010 M).

8. Selenium Stock Solution. Dissolve 0.173 g Na_2SeO_3 in a 1-liter volumetric flask and fill to the mark with Q water. Rinse a 250-mL LPE bottle with the stock solution then fill the bottle (Se_T = 0.001 M).

9. Zinc Stock Solution. Dissolve 0.136 g $ZnCl_2$ in a 1-liter volumetric flask and fill to the mark with Q water. Rinse a 250-mL LPE bottle with the stock solution then fill the bottle (Zn_T = 0.001 M).

Do not pipette Trace Element Stock Solutions directly from LPE storage bottles. Rinse a 50-mL LPE bottle with stock solution. Pour a few mL of stock solution into the smaller bottle, then pipette from the smaller bottle. If stock solution contamination is even remotely suspected, reprepare that solution.

Appendix I (continued)

Fe-EDTA Stock Solution.

The chelating agent EDTA (ehtylenediaminetetraacetic acid) is used in Aquil to prevent ferric hydroxide formation. Precipitation of ferric hydroxide is indicated by a rust-colored film on the Millipore filter. Stock solution is prepared as follows:

1. add 0.270 g $FeCl_3 \cdot 6H_2O$ and 6.217 g Na_2EDTA to approximately 900 mL Q water,
2. stir solution and adjust pH to 8.1 $\pm$ 0.1 with spectrograde Na_2CO_3 and HCl,
3. after salts have dissolved, pour solution into a 1-liter volumetric flask and fill to the mark with Q water (Fe_T = 0.001 M, $EDTA_T$ = 0.020 M).

Appendix I (continued)

Trace Element Spiking Levels.

Spiking volumes are summarized in the following table for addition to the 2-liter Teflon storage bottle. Use Trace Element Stock Solutions as described above.

Stock	Add	for every
Arsenic	20 µL	0.1 µM desired
Cobalt	20 µL	0.01 µM desired
Copper	20 µL	0.01 µM desired
Iodine	20 µL	0.01 µM desired
Iron	20 µL	0.01 µM desired
Manganese	20 µL	0.01 µM desired
Molybdenum	20 µL	0.10 µM desired
Selenium	20 µL	0.01 µM desired
Zinc	20 µL	0.01 µM desired.

Trace element solutions are added with one of three autopipettes. For instructions on the use of these autopipettes, refer to the equipment specifications section (Section XI).

APPENDIX IA

IONIC STRENGTH EFFECTS ON MEASURED pH

Background.

Glass pH electrodes measure activity of the hydrogen ion not its concentration. Concentration of any ion may be related to its activity through an activity coefficient f. In the case of the hydrogen ion, $\{H^+\} = f[H^+]$. If one defines $p_cH \equiv -\log[H^+]$, and $p_aH \equiv -\log\{H^+\} = pH_{measured}$, then $p_aH = p_cH - \log f$ or $p_cH = pH_{measured} + \log f$.

The Davies Equation (Stumm and Morgan, 1970) may be used to obtain an estimate for log f:

$\log f = -AZ^2 \left(\left(I^{\frac{1}{2}}/(1+I^{\frac{1}{2}}) \right) - 0.3I \right)$, where I equals ionic strength, A = a constant = 0.51 at 29°C (Bates, 1973), and Z is the ion charge. For I = 0.5 M, log f = -0.14. This calculations reveals that measured pH will be higher than p_cH by approximately 0.14 units for I = 0.5 M at 29°C solution temperature.

Procedure.

An experiment was run to determine a log f value using an Aquil-type matrix. Tris buffers were prepared at pH = 8.0 and 8.4 with I = 0.5 M set by NaCl and $MgCl_2 \cdot 6H_2O$ (the two largest constituents of the Aquil Major Salts Solution).

pH and emf measurements were taken using an Orion 801A ion analyzer and a Broadly-James combination pH electrode.

Appendix IA (continued)

The electrode was initially calibrated with commercial buffers at pH = 7.0 and 10.0. Tris buffers at I = 10^{-3} M were then measured against Tris buffers at I = 0.5 M to observe ionic strength effects (Table B).

Discussion.

The data indicate close agreement between predicted and observed estimates for -log f (0.14 to 0.135 respectively). Possible sources of error would include:

1. errors in preparing the Tris buffers,
2. volume effects due to temperature variations of solutions, and
3. accuracy limitations in weighing Tris buffer salts.

The log f value determined in this experiment and that determined by Bates (1973) for an artificial seawater medium, I = 0.66 M were nearly identical (0.135 to 0.136 respectively). Those preparing and using Aquil should be aware of this pH shift.

Appendix IA (continued)

Table B. Ionic strength effects on pH_m*.

Tris Buffer Sample	pH_m	$\overline{pH}_m$
Specified pH = 8.0 ($I = 10^{-3}$ M)	7.88	
	7.89	
		7.90
	7.91	
	7.91	
Specified pH = 8.4 ($I = 10^{-3}$M)	8.29	8.29
	8.28	
Specified pH = 8.0 ($I = 0.5$ M)	8.01	
	8.01	
		8.02
	8.03	
	8.03	
Specified pH = 8.4 ($I = 0.5$ M)	8.44	8.44
	8.44	

Estimates for -log f:

$$pH_{I=0.5M} - pH_{I=10^{-3}M} = 8.02 - 7.90 = 0.12 \text{ (at pH = 8.0)},$$

$$= 8.44 - 8.29 = 0.15 \text{ (at pH = 8.4)}.$$

*Measurements taken by J. Faughnan on 7/25/79. pH values rounded off to three significant figures. Temperature range in the Cleanroom was 28.5 to 30.0°C.

APPENDIX IB

TEMPERATURE EFFECTS ON HYDROGEN ION ACTIVITY IN AQUIL

Background.

Activity of hydrogen ions $\{H^+\}$ is temperature dependent. By taking the derivative with respect to temperature of the Conservation of Mass equation for a solution of salt MHB of the dibasic acid H_2B, with dissociation constants K_1 and K_2, Bates (1973) determined a relationship for the effect of temperature on hydrogen ion activity:

$$\frac{-\partial \log \{H^+\}}{\partial T} \simeq -1/2 \frac{\partial \log K_1}{\partial T} - 1/2 \frac{\partial \log K_2}{\partial T} + 2 \frac{\partial \log \gamma}{\partial T}$$

where γ = mean activity coefficient for the matrix, and T in °K.

The first two terms in the equation can be evaluated using the van't Hoff equation:

$$\frac{\partial \ln K}{\partial T} = \frac{\Delta H^\circ}{RT^2}$$

At 22°C: $\frac{\partial \log K_1}{\partial T} = 0.0055/°K$ and $\frac{\partial \log K_2}{\partial T} = 0.0088/°K$ (for the carbonate system).

An estimate for the final term in Bates' equation was given by Harned and Owen (1958):

$$\frac{\partial \log \gamma}{\partial T} = -0.00014/°K \text{ (at 22°C)}.$$

Combining the three values gives:

$\frac{-\partial \log \{H^+\}}{\partial T} \cong \frac{\Delta pH_m}{\Delta T}$ = variation of the measured pH due to temperature = $-1/2(0.0055) -1/2(0.0088) + 2(-0.00013) = -0.0074/°K$.

For example, if this solution is cooled by 15°C, an increase in pH_m of approximately 15(0.0074) = 0.11 units results.

Appendix IB (continued)

Procedure.

An experiment was conducted to measure temperature effects on measured pH (pH_m) for the two pH electrodes used in Aquil preparation (i.e., Fleximark 2885 and Broadly-James combination electrodes). Measurements using both electrodes were made over a temperature range of 10 to 30°C using an Orion 801A ion analyzer (Table C, Figure C). The sample used for measurement was pH adjusted Aquil prior to trace element and macronutrient addition.

Discussion.

Predicted and measured magnitudes of this temperature shift in activity compared favorably for both electrodes (0.11 and 0.13 units respectively). The 0.02 unit discrepancy may be due to the approximation for $\partial \log \gamma / \partial T$ in the mathematical prediction. Bates (1973) indicates a value of -0.009/°K for $\partial \log \{H^+\} / \partial T$ in a 0.025 M bicarbonate solution. Using this value and correcting for ionic strength effects, the variation predicted for a 15°C temperature change is 0.14 units.

In summary, if one adjusts Aquil pH to 8.10 in the Cleanroom (29°C), then pH_m will increase to 8.23 when the solution is used at 14°C (a typical culturing temperature). However, due to the high ionic strength of Aquil, p_cH will be 0.13 units lower than pH_m (see Appendix IA). The two effects cancel one another in this case.

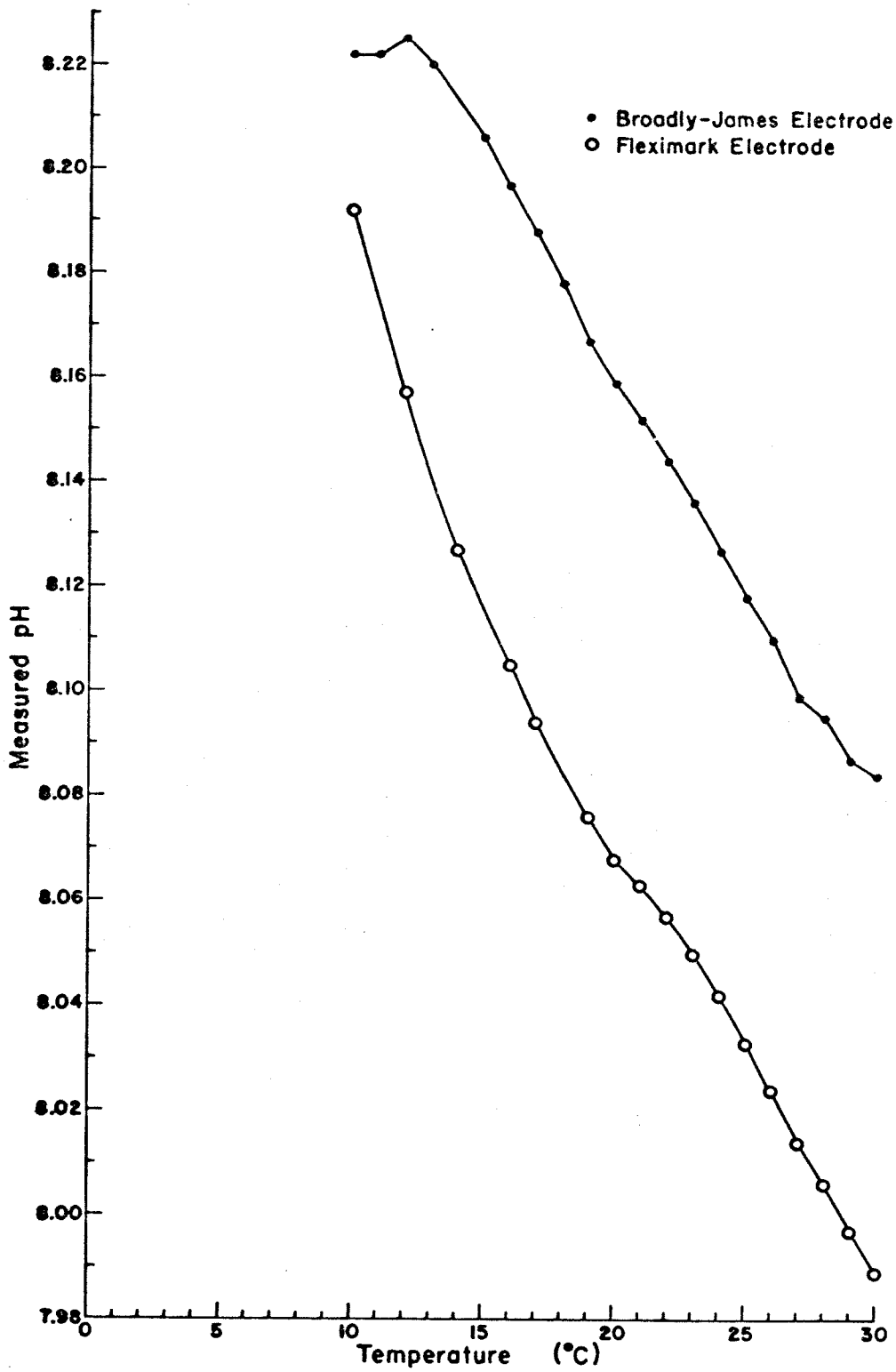
Appendix IB (continued)

Table C. Temperature Effects on Measured pH*.

Temperature (°C)	Measured pH Broadly-James	Fleximark
10	8.222	8.192
11	8.222	
12	8.225	8.156
13	8.220	
14	8.213	8.127
15	8.206	
16	8.197	8.105
17	8.188	8.094
18	8.178	
19	8.167	8.076
20	8.159	8.068
21	8.152	8.063
22	8.144	8.057
23	8.136	8.050
24	8.127	8.042
25	8.118	8.033
26	8.110	8.024
27	8.099	8.014
28	8.095	8.006
29	8.087	7.997
30	8.084	7.989

*Data collected by J. Faughnan, 7/30/79.

Figure C. Temperature effect on measured hydrogen ion activity (pH_m).



APPENDIX II

TEMPERATURE CORRECTIONS FOR REDEQL2 ANALYSIS.

Gametophyte culturing experiments were run between 10 and 18°C while equilibrium constants are typically determined at 25°C (20°C for EDTA complexes). It was therefore of interest to determine effects of temperature on speciation of Aquil components. Computer subroutine TEMCOR was incorporated into the REDEQL2 program to estimate these effects. The following report was submitted to inform other REDEQL2 users of the creation and application of TEMCOR.

APPENDIX II

TEMPERATURE CORRECTIONS FOR REDEQL2 ANALYSIS.

Background.

Equilibrium constants are influenced by temperature (Stumm and Morgan, 1970). A subroutine called TEMCOR has been incorporated into the REDEQL2 program file to estimate this influence using the integrated van't Hoff Equation. The correction equation is derived below from an expression for the free energy of a reaction and from the van't Hoff Equation.

$$RT \ln K = -\Delta G^{o} = T\,\Delta S^{o} - \Delta H^{o} \quad \text{(Free energy of a reaction)}.$$

$$\ln K = \frac{\Delta S^{o}}{R} - \frac{\Delta H^{o}}{RT}$$

Assuming ΔS^{o} and ΔH^{o} are independent of temperature:

$$\frac{d}{dT}(\ln K) = -\frac{\Delta H^{o}}{R}(-T^{-2}) \quad \text{(van't Hoff Equation)}.$$

Integrate with respect to T.

$$\ln K \Big|_{K(T_1)}^{K(T_2)} = \int_{T_2}^{T_1} \frac{\Delta H^{o}}{R}(T^{-2})\,dT \quad \text{or}$$

$$\log K(T_2) = \log K(T_1) + \frac{\Delta H^{o}}{2.303R}(T_1^{-1} - T_2^{-1}) \quad \text{(eq. *)}.$$

After equilibrium constants are corrected for ionic strength, eq. * is applied by subroutine TEMCOR. The necessary input parameters are:

1. $H^{o} \equiv H$ = Enthalpy change for the formation reaction (Kcal/mole),

Appendix II (continued)

2. Referencetemperature ≡ TR = Temperature at which formation constant (in THERMO) was determined. If unknown, TR defaults to 25°C.
3. T ≡ Temperature at which analysis is desired (°C).

Input parameters are indexed identically to equilibrium constants in THERMO (the thermodynamic data set).

Refer to the attached example of an analysis of Luigi Provasoli's synthetic seawater (1953). Note that only the FORT DD * card and subroutine source deck are inserted within the original program deck. If temperature corrections are not desired, submit the program deck as done before (a dummy subroutine is then loaded).

References for existing input data for TEMCOR are attached on Tables D-F. Comment statements have been inserted into the TEMCOR source deck for user convenience and information.

Original REDEQL2 Program Deck.

```
//PROVAS JOB (61601,JSK,EES),KUWABARA
/*ROUTE  PRINT R3
/*JOBPARM REGION=320K,LINES=3
//       EXEC  FORTG
//SYSLIB    DD
//          DD
//          DD DSN=JBM.REDEQL2,UNIT=SYSDA,
//             VOL=SER=CITSL1,DISP=SHR
//FT10F001   DD  UNIT=SYSDA,DSN=JSK.THERMO,
//             VOL=SER=CITSL1,DISP=SHR
//FORT   DD     *
//LOAD   DD     *          <-- Insert TEMCOR source deck here.
 INCLUDE SYSLIB,REDEQL
 ENTRY  MAIN
//DATA   DD     *
 11 13  1  2  0  0  0  0  0  0  0  0  0  0  0  0
 7.00-1
 1     2.04  1.98
 2     1.33  1.26
 3     4.20  4.20
 4     2.00  1.99
 5     0.33  0.32
 6    18.00  5.26
 8     8.00  5.49
 9    14.00  7.80
12    11.00  6.60
16    10.00  6.21
 1     4.34  2.62
 2     1.90  1.54
 3     0.25  0.25
 4     4.38  4.15
 5     3.08  3.08
 9     8.84  4.68
28    13.00  4.79
48     4.50  4.21
52     8.82  8.82
54     8.00  8.00
56     8.25  8.00
57     3.26  3.26
       7.9
 3.5
 1 9-1 2 9-1
//
```

Subroutine TEMCOR Source Listing.

```
      SUBROUTINE TEMCOR
C-----THE FOLLOWING SUBROUTINE MAKES TEMPERATURE CORRECTIONS ON
C-----EQUILIBRIUM CONSTANTS USING AVAILABLE ENTHALPY DATA.
      DIMENSION H(20,30,6), TR(20,30,6),SH(20,30,6)
      COMMON/ICST/NM,NL,NM1,NM2,NM3,NL1,NL2,NL3,NMADS,NLADS,NEQ,NIQ,NML,
     1NSOL,NPEDOX,ITER,NMIX,NBMIX,NC,NS,NCASES,ITMAX,INREX,I0,I7,IAT,I8
     2,ICON
      COMMON/ICOF/CM(20,30,6),CL(20,30,6),CHOH(20,30,6),CK(20,30,6),KSM(
     120,30,3),KSL(20,30,3),KSHOH(20,30,3),SOLHY(20,30,3),ISOLUB(20,30)
      INTEGER*2 CM,CL,CHOH,CK,KSM,KSL,KSHOH,SOLHY,ISOLUB,LO,LI,MO,MI,IDU
     1M,JDUM,KRED,NELEC,NHRED,REDCST,KMTX,MIX1,MIX2,LIX1,LIX2,KSMIX1,KSM
     1IX2,KSLIX1,KSLIX2,KSHIX,SOLIX,MREDOX,LREDOX,KSAL,KSSI,CHGM,CHGL
      INTEGER*2 KDEL,KSDEL
C-----INPUT THE TEMPERATURE OF THE MEDIA IN DEGREES CENTIGRADE.
      T = 14.0
      R = 1.987E-03
C-----INPUT DEFAULT ENTHLAPY AND TEMPERATURE VALUES.
      DO 20 I= 1,NM
      DO 20 J= 1,NL
      DO 20 K= 1,6
      H(I,J,K) = 0.0
      SH(I,J,K) = 0.0
      TR(I,J,K) = 25.0
   20 CONTINUE
C-----DELTA H AND TEMPERATURE DATA ARE INPUT HERE.
      SH(1,1,1) = 3.1
      H(1,2,1) = 1.7
      H(2,2,1) = 4.9
      H(2,6,1) = -0.2
      H(2,13,1) = 2.1
      H(4,2,1) = 3.1
      H(5,2,1) = 2.2
      H(6,3,1) = 7.9
      H(6,3,2) = 8.0
      H(6,3,3) = 10.3
      SH(6,13,1) = -25.6
      H(7,2,1) = 3.4
      H(7,3,1) = 8.0
      H(8,3,1) = 8.7
      H(8,3,2) = 10.6
      H(8,3,3) = 13.7
      H(8,3,4) = 17.8
      H( 9,3,1) = 7.8
      H( 9,3,2) = 8.5
      H( 9,3,3) = 9.6
      H( 9,3,5) = 11.0
```

Subroutine TEMCOR Source Listing (continued).

```
      H( 9,2,1) = 4.0
      H(10,2,1) = 1.7
      H(10,3,1) = 8.0
      H(11,1,1) = -3.6
      H(11,1,2) = -5.4
      H(11,2,1) = 4.9
      H(11,6,1) = -3.5
      H(11,6,2) = -4.5
      H(1, 7,1) = -6.5
      H(2, 7,1) = 3.2
      H(3, 7,1) = -4.1
      H(5, 7,1) = -2.5
      H(6, 7,1) = -2.5
      H(7, 7,1) = -4.6
      H(8, 7,1) = -8.2
      H( 9, 7,1) = -4.?
      H(10, 7,1) = -4.?
      H(11, 7,1) = -5.7
      H(11, 7,2) = -4.4
      H(11, 7,3) = 1.4
      H(11, 7,4) = 0.2
      TR( 1, 7,1) = 20.0
      TR( 2, 7,1) = 20.0
      TR( 3, 7,1) = 20.0
      TR( 5, 7,1) = 20.0
      TR( 6, 7,1) = 20.0
      TR( 7, 7,1) = 20.0
      TR( 8, 7,1) = 20.0
      TR( 9, 7,1) = 20.0
      TR( 10,7,1) = 20.0
      TR( 11,7,1) = 20.0
      TR( 11,7,2) = 20.0
      TR( 11,7,3) = 20.0
      TR( 11,7,4) = 20.0
C-----TEMPERATURE (T) IS CONVERTED INTO KELVIN SCALE.
      T = T + 273.0
C-----THE FOLLOWING LOOPS CORRECT CONSTANTS FOR TEMPERATURE.
C-----CORRECTIONS MADE FOR EQUILIBRIUM CONSTANTS CK.
      DO 32 I= 1,NM
      DO 31 J= 1,NL
      DO 30 K= 1,6
```

Subroutine TEMCOR Source Listing (continued).

```
      IF(H(I,J,K).EQ.0.0)GO TO 30
      KDEL = 100.0 * ((H(I,J,K)/(2.303*R)) *
     1((1.0/(TR(I,J,K)+273.0)) - (1.0/T)))
      CK(I,J,K) = CK(I,J,K) + KDEL
   30 CONTINUE
   31 CONTINUE
   32 CONTINUE
C-----CORRECTIONS MADE FOR DISSOLUTION CONSTANTS SOLHY.
      DO 42 I=1,NM
      DO 41 J=1,NL
      DO 40 K=1,3
      IF(SH(I,J,K).EQ.0.0)GO TO 40
      KSDEL = 100.0 * ((SH(I,J,K)/(2.303*R)) *
     1((1.0/(TR(I,J,K)+273.0)) - (1.0/T)))
      SOLHY(I,J,K) = SOLHY(I,J,K) + KSDEL
   40 CONTINUE
   41 CONTINUE
   42 CONTINUE
      RETURN
      END
```

TABLE D. STABILITY CONSTANTS OF EDTA COMPLEXES IN AQUIL[1]

	$\log K_{ML}$ (20°C)[2]	$\log K_{MHL}$ (20°C)	ΔH (Kcal/mole)	$\frac{\Delta \log K_{ML}}{\Delta T}$[3]	$\log K_{ML}$ (14°C)
Ca^{++}	10.7	3.5	-6.5	-0.017	10.6
Mg^{++}	8.7	2.3	3.2	0.008	8.7
Sr^{++}	8.6	2.3	-4.1	-0.010	8.5
K^{+}	0.8	-0.3	—[4]	—	
Na^{+}	1.7	-0.03	-2.5	-0.006	1.6
Fe^{+++}	25.1	1.2	—	—	
Mn^{++}	14.0	3.1	-4.6	-0.012	13.9
Cu^{++}	18.9	3.0	-8.2	-0.021	18.7
Zn^{++}	16.7	3.0	-4.9	-0.012	16.6
Co^{++}	16.5	3.0	-4.2	-0.011	16.4

[1] Anderegg, 1977.

[2] K_{ML}: $M^{q} + L^{-4} \rightleftarrows ML^{q-4}$

K_{MHL}: $M^{q} + HL^{-3} \rightleftarrows MHL^{q-3}$

[3] Estimated using van't Hoff Equation

[4] — = not available (ie., K_{KL} value not corrected for temperature.)

TABLE E. PROTONATION CONSTANTS FOR EDTA[1]

	$\log_{10} K$ (20°C)	ΔH (Kcal/mole)	$\frac{\Delta \log K}{\Delta T}$ (°C^{-1}) [2]	$\log_{10} K$ (14°C)
K_1 [3]	10.23	-5.69	-0.014	10.14
K_2	6.18	-4.39	-0.011	6.11
K_3	2.66	1.43	0.004	2.68
K_4	2.01	0.18	0.0005	2.01

[1] Anderegg, 1977.

[2] Estimated using van't Hoff Equation.

[3] K_p: $H_{p-1}L^{(p-3)} + H^+ \rightleftarrows H_pL^{(p-4)}$.

Table F. Enthalpy Data for Some Aquil Reactions (25°)

Reaction	ΔH (kcal/mole)	Reference
$Ca^{++} + CO_3^{=} = CaCO_3$	3.1	[1]Helgeson (1969)
$Ca^{++} + SO_4^{=} = CaSO_4$	1.7	[2]Nancollas (1966)
$Mg^{++} + SO_4^{=} = MgSO_4$	4.9	[3]Truesdell and Jones (1974)
$Mg^{++} + HPO_4^{=} = MgHPO_4$	3.3	3
$Mg^{++} + H^{+} + PO_4^{\equiv} = MgHPO_4$	-0.2	3
$Mg^{++} + OH^{-} = MgOH^{+}$	2.1	1
$K^{+} + SO_4^{=} = KSO_4$	3.1	3
$Na^{+} + SO_4^{=} = NaSO_4$	2.2	3
$Fe^{+3} + Cl^{-} = FeCl^{++}$	7.9	1
$Fe^{+3} + 2Cl^{-} = FeCl_2^{+}$	8.0	1
$Fe^{+3} + 3Cl^{-} = FeCl_3$	10.3	1
$Fe^{+3} + 4Cl^{-} = FeCl_4^{-}$	13.9	1
$Fe + 3OH^{-} = FeOOH + H_2O$	-25.56	3
$Mn^{++} + SO_4^{=} = MnSO_4$	3.4	2
$Mn^{++} + Cl^{-} = MnCl^{+}$	8.0	Estimated from 1
$Cu^{+2} + Cl^{-} = CuCl^{+}$	8.7	1
$Cu^{+2} + 2Cl^{-} = CuCl_2$	10.6	1
$Cu^{+2} + 3Cl^{-} = CuCl_3^{-}$	13.7	1
$Cu^{+2} + 4Cl^{-} = CuCl_4^{=}$	17.8	1

Table F (continued) Enthalpy Data for Some Aquil Reactions (25°)

Reaction	ΔH (kcal/mole)	Reference
$Zn^{+2} + Cl^- = ZnCl^+$	7.8	1
$Zn^{+2} + 2Cl^- = ZnCl$	8.5	1
$Zn^{+2} + 3Cl^- = ZnCl^-$	9.6	1
$Zn^{+2} + 4Cl^- = ZnCl^{=}$	11.0	1
$Zn^{+2} + SO_4^{=} = ZnSO_4$	4.0	2
$Co^{++} + Cl^- = CoCl^+$	8.0	Estimated from 1
$Co^{++} + SO_4^{=} = CoSO_4$	1.7	2
$H^+ + CO_3^{=} = HCO_3^-$	-3.6	1
$2H^+ + CO_3^{=} = H_2CO_3$	-5.4	1
$H^+ + SO_4^{=} = HSO_4^-$	4.9	2
$H^+ + PO_4^{\equiv} = HPO_4^{=}$	-3.5	2
$2H^+ + PO_4^{\equiv} = H_2PO_4^-$	-4.5	2

APPENDIX III.

NUTRIENT DIFFUSION COMPUTATIONS.

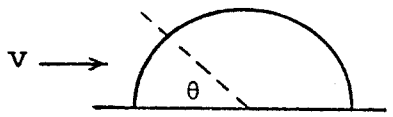
In using the initial trough culturing system, it was observed that an average media velocity exceeding 1 cm/sec. caused settled spores to be scoured off the culture slides (both in the center and sides of the channel). Due to this restriction on average velocity, the possibility of a diffusion limiting condition in the initial trough culturing design was considered.

APPENDIX III

NUTRIENT DIFFUSION COMPUTATIONS

Diffusion of chemical nutrients to a Macrocystis gametophyte was estimated using the following assumptions:

1. a cell is a hemisphere of radius R_c, and
2. that the problem can be modeled as a solution with bulk concentration c_b moving past a hemisphere at velocity v.



Applying Fick's First Law: $\partial w/\partial t = DS(\partial c/\partial x)$, the flux through a diffusion layer of thickness $\delta(\theta)$ on a differential lune of angle $d\theta$ is given by:

$$\frac{\partial w}{\partial t}\Big|_{\theta}^{\theta+d\theta} = \int_{\theta}^{\theta+d\theta} DS_{dL}\,\frac{(c_o - c_b)}{\delta(\theta)}$$

where: D = nutrient (ion) diffusion coefficient, θ = angle in relation to the flow, and S_{dL} = surface area of the differential lune.

Diffusion flux over the hemisphere is given by:

$$\frac{\partial w}{\partial t}\Big|_{0}^{\pi} \text{ rate (J)} = \int_{0}^{\pi} D(2R_c^{\,2}d\theta)\,\frac{(c_o - c_b)}{\delta(\theta)} \quad \text{where;}$$

$$\delta(\theta) = \frac{1}{\sqrt{3/\pi}}\,(R_c D)^{\frac{1}{2}} v^{-\frac{1}{2}}\,(2+\cos\theta/\,(1+\cos\theta)^2)^{\frac{1}{2}} \quad \text{(Levich, 1962)}$$

$$\text{or } J = 2\sqrt{3/\pi}\,D^{\frac{1}{2}}R_c^{\,3/2}\,v^{\frac{1}{2}}\,(c_o - C_b)\,\{\underbrace{\int_0^{\pi}\frac{1+\cos\theta}{(2+\cos\theta)^{\frac{1}{2}}}\,d\theta}\}$$

Call this I.

NUTRIENT DIFFUSION COMPUTATIONS (continued)

Let: $D = 10^{-5}$ cm^2/sec (Robinson, 1959)

$R_c = 5 \times 10^{-4}$ cm

$c_o = 0$ (ie., cell wall is perfect sink)

c_b = Concentration in growth medium.

$\bar{u}$ = average velocity = 1 cm/sec.

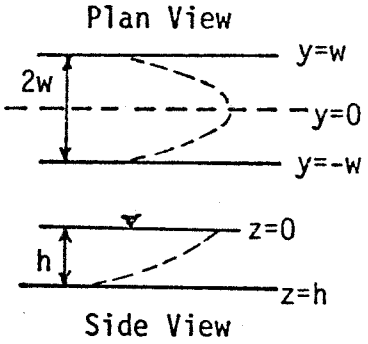
Note: 1. Laminar conditions exist: $R = v(2R_c)/\nu \leq \bar{u}(2R_c)/\nu$

$$= \frac{1 \text{ cm/sec } (2)(5\times10^{-4} \text{ cm})}{1.3 \times 10^{2} \text{ cm}^2/\text{sec}}$$

$$= 0.08 < 1.$$

2. $\delta(90°) = 22$ microns.

The following approximation was used to estimate v.



Assuming a velocity distribution of the form:

$$u(y,z) = u_{max} \left(1 - \left(\frac{z}{h}\right)^2\right)\left(1 - \left(\frac{y}{w}\right)^2\right)$$

The average velocity $\bar{u}$ is given by:

$$\bar{u} = \frac{1}{A} \int_0^h \int_0^w u(y,z)\, dy\, dz = \frac{1}{2wh} (2/3\ h)(4/3\ w)\ u_{max} = 4/9\ u_{max}$$

For $\bar{u} = 1$ cm/sec, $u_{max} = 2.25$ cm/sec.

Let $v = u\left(\frac{w}{2}, h - \frac{R_c}{2}\right) = 2.1\times10^{-3}$ cm/sec.

Then $J = 2\sqrt{3/\pi}\ (10^{-2.5})(5\times10^{-4})^{3/2}(2.1\times10^{-3})^{1/2}\ (-c_b)(I)$

$I = 2.01$ (numerical solution)

$$= -6.4\times10^{-9} \text{ cm}^3/\text{sec } (c_b)$$

or $J = -2.3\times10^{-8}$ liter/day (c_b).

Appendix III (continued)

TABLE G. DIFFUSION OF CERTAIN NUTRIENTS INTO GAMETOPHYTE CELLS.

Nutrient	Typical Free Ion Activity*	Computed Diffusion Rate (moles/day)**	Computed Uptake Rate (moles/day)***
Manganese	10^{-9}	2×10^{-17}	4×10^{-17}
Iron	10^{-19}	2×10^{-27}	7×10^{-16}
Cobalt	10^{-10}	2×10^{-18}	2×10^{-17}
Copper	10^{-11}	2×10^{-19}	1×10^{-17}
Zinc	10^{-9}	2×10^{-17}	5×10^{-16}
CO_2	10^{-5}	2×10^{-13}	2×10^{-13}

*Kester, 1974.

**Diffusion model.

***Trace element data by North (1977).
Oxygen evolution data by P.A. Wheeler (personal communication).

APPENDIX IV

METAL-EDTA COMPLEXATION AND METAL UPTAKE IN AQUIL CULTURES

The uptake rate of a metal, Me, in Aquil by Macrocystis gametophytes was estimated by applying the following assumptions:

1. Concentration of a metallic micronutrient, Me, in Macrocystis is 10^{-7} moles Me/g(wet) (estimated from juvenile sporophyte data [North, 1977a]),
2. Me_T = 10 nM of which 99% is complexed by EDTA (see Chapter 6),
3. Gametophytes are attached to microslides (3"x1") at a density of 100 gametophytes/mm^2 (see Chapter 4),
4. Gametophytes are batch cultured in 250 mL Aquil using 2 microslides per culturing vessel (see Chapter 4),
5. Cell density (mass/unit cell volume) can be approximated be seawater density ($\sim$1 g/cm^3),
6. After 14 culturing days, gametophytes can be modeled as cylinders with radius, R_c = 10 microns and length l_c = 150 microns (see Chapter 6). Then gametophyte volume is approximately 5×10^{-8} cm^3.

Estimated Me uptake rate, j, in an Aquil batch culture was therefore:

$$j = (100 \text{ gametophytes/mm}^2)(3 \text{ in}^2\text{/microslide})(25.4^2 \text{ mm}^2\text{/in}^2) \times (2 \text{microslides/culturing vessel})(5\times10^{-8} \text{ cm}^3\text{/gametophyte-14 days})(1 \text{ g/cm}^3)(10^{-7} \text{ moles Me/g(wet)})(\text{culturing vessel/250 mL})(10^3 \text{ mL/liter})(\text{day/24x3600 sec}) \cong 6\times10^{-15} \frac{\text{moles Me}}{\text{liter-sec}}.$$

APPENDIX IV (continued)

Dissociation rates for the Me-EDTA complexes in Aquil ($\sim 10^{-10}$ moles Me/liter-sec), estimated from kinetics data by Margerum (1962), are significantly faster then the computed metal uptake by Macrocystis gametophytes.

REFERENCES

Adriano, D. C., G. M. Paulsen and L. S. Murphy, 1971, Phosphorous-iron and phosphorous-zinc relationships in corn (Zea mays L.) seedlings as affected by mineral nutrition. Agron. J., 63, 32-39.

Ahrland, S., 1975, Metal complexes present in seawater, in The Nature of Seawater, E. D. Goldberg, ed., Dahlem Konferenzen, Berlin, Germany, 719 pp.

Alberts-Dietert, F., 1941, Die Wirkung von Eisen und Mangan auf die Stickstoffassimilation von Chlorella, Plant., 32, 28-117.

Allen, E. J., 1914, On the culture of planktonic diatom Thalassiosira gravida, in artificial seawater, J. Mar. Biol. Assoc. U. K., 10, 417-439.

Anderegg, G., 1977, Critical Survey of Stability Constants of EDTA Complexes, Pergamon Press, N. Y., 42 pp.

Anderson, D. M. and F. M. M. Morel, 1978, Copper sensitivity of Gonyaulax tamarensis, Limnol. Oceanogr., 23, 283-295.

Balistrieri, L. S., 1977, The basic surface characteristics of goethite (α- FeOOH), M. S. Dissertation, Univ. of Washington, Seattle, WA, 65 pp.

Barber, R. T. and S. A. Huntsman, 1978, Copper ion activity: The inherent toxic factor in natural deep ocean seawater, in Workshop on Copper in Estuarine, Continental and Marine Waters, U. S. Dept. of Energy, Washington, D. C., 36 pp.

Bates, R. G., 1973, Determination of pH, Theory and Practice, second ed., Wiley Interscience, N. Y., 479 pp.

Brewer, P. G., 1975, Minor Elements in seawater, in Chemical Oceanography, Vol. 1, Chap. 7, Academic Press, London, 642 pp.

Brown, J. C., J. E. Ambler, R. L. Chaney and C. D. Foy, 1972, Differential responses of plant genotypes to micronutrients, in Micronutrients in Agriculture, J. J. Mortvedt, P. M. Giordaro and W. L. Lindsay, eds., Soil Science of America, Inc., Madison, WI, 666 pp.

REFERENCES (CONT.)

Buetow, D. E., 1962, Differential effects of temperature on the growth of Euglena gracilis, Expl. Cell Res., 27, 137-142.

Bunt, J. S., 1965, Measurement of photosynthesis and respiration in a marine diatom (Fragilaris sublinearis) with the mass spectrometer and with carbon-14, Nature, London, 207, 1373-1375.

Cheniae, G. M. and I. F. Martin, 1969, Photoreactivation of manganese catalyst in photosynthetic oxygen evolution, Pl. Physiol., Lancaster, 44, 351-360.

Chesire, M. F., P. C. DeKock and R. H. E. Inkson, 1967, Factors affecting the copper content of oats grown in peat, J. Sci. Food Agr., 18, 156-160.

Chu, S. P., 1942, The influence of the mineral composition of the medium on the growth of planktonic algae, J. Ecol., 30, 284-325.

Coleman, J. E. and B. L. Vallee, 1964, Metallocarboxypeptidase inhibitor complexes, Biochem., 3, 1874-1879.

Corredor, J. E., 1979, Phytoplankton response to low level nutrient enrichment through upwelling in the Columbian Caribbean Basin. Deep Sea Research, 26(7), 731-742.

Davey, E. W., M. J. Morgan and S. J. Erickson, 1973, A biological measurement of copper complexation capacity of seawater, Limnol. Oceanogr., 18, 993-997.

Davis, J. A. and J. O. Leckie, 1978, Surface ionization and complexation at the oxide/water interface, J. Colloid and Interface Science, 67, 90-107.

Devinney, J. S. and L. A. Volse, 1978, Effects of sediments on the development of Macrocystis pyrifera, Mar. Biol., 48, 343-348.

Eilers, H., 1926, Zur Kenntnis der Ernährungsphysiologie von Stichococcus bacillaris Näg., Rec. Trav. Botan. Neerl., 23, 362-395.

Emerson, R. and C. M. Lewis, 1939, Factors affecting the efficiency of photosynthesis, Am. J. Bot., 26, 802-822.

Eyster, C., T. E. Brown and H. A. Tanner, 1953, Mineral requirements for Chorella pyrenoidosa under autotrophic and heterotrophic conditions, in Trace Elements, C. A. Lamb, O. G. Bentley and J. M. Beattie, eds., Academic Press, N. Y., 274 pp.

REFERENCES (CONT.)

Finney, D. J., 1960, The Theory of Experimental Design, University of Chicago Press, IL, 223 pp.

Giesy, J. P., 1976, Stimulation of growth in Scenedesmus obliquus (Chlorophyceae) by humic acids under limited conditions, J. Phycol., 12, 172-179.

Grimme, H., 1968, Die Adsorption von Mn, Co, Cu und Zn durch Goethit aus verdünnten Lösungen, Z. Planzern, Düng, Bodenk, 121, 58-65.

Haines, K. C. and P. A. Wheeler, 1978, Ammonium and nitrate uptake by the marine macrophytes Hypnea musciformis (Rhodophyta) and Macrocystis pyrifera (Phaeophyta), J. Phycol., 14, 319-324.

Hanger, B. C., 1965, The influence of iron upon the toxicity of manganese, molybdenum, copper, and boron in red clover (Trifolium pratense L.), J. Aust. Inst. Agr. Sci., 31, 315-317.

Harned, H. S. and B. B. Owen, 1958, The Physical Chemistry of Electrolytic Solutions, Reinhold Publ. Corp., third ed., 645 pp.

Harvey, H. A., 1937, The supply of iron to diatoms, J. Mar. Biol. Assoc. U. K., 22, 205-219.

Harvey, H. W., 1947, Manganese and the growth of phytoplankton, J. Mar. Biol. Assoc. U. K., 26, 562-579.

Helgeson, H. C., 1969, Thermodynamics of hydrothermal systems at elevated temperatures and pressures, Am. J. Sci. 267, 729-804.

Hidy, G. M., 1974, Characterization of aerosols in California, final report, State of California Air Resources Board, pp. 3-1 to 3-44.

Holm-Hansen, O., G. C. Gerloff and F. Skoog, 1954, Cobalt as an essential element for blue-green algae, Physiol. Plantarum, 7, 665-675.

Hopkins, E. F., 1930, Manganese, an essential element for a green alga, Am. J. Bot., 17, 1047-1051.

REFERENCES (CONT.)

Hutner, S. H., S. Aaronson, H. A. Nathan, H. Baker, S. Scher, and A. Cury, 1958, Trace elements in microorganisms; the temperature factor approach, in Trace Elements, C. A. Lab, O. G. Bentley and J. M Beattie, eds., Academic Press, N. Y., 274 pp.

Jackson, G. A., 1977, Nutrients and production of giant kelp, Limnol. Oceanogr., 22, 979-995.

Jorgensen, E. G., 1962, Antibiotic substances from cells and culture solutions of unicellular algae with special reference to some chlorophyll derivatives, Physiol. Plantarum, 15, 530-545.

Katoh, S., 1960, A new copper protein from Chlorella ellipsoidea, Nature, 186, 533-534.

Kester, D. R., et al., 1975, Chemical speciation in seawater, in The Nature of Seawater, E. D. Goldberg, ed., Dahlem Konferenzen, Berlin, Germany, 719 pp.

Kirkby, E. A. and K. Mengel, 1967, Ionic balance in different tissues of the tomato plant in relation to nitrate, urea or ammonium nutrition, Plant Physiol., 42, 6-14.

Knauer, G., 1978, Low level effects of seawater Cu, Zn, and Mn on marine phytoplankton, Southern California Coastal Water Research Project seminar, Los Angeles, CA.

Krauss, R. N., 1955, Nutrient supply for large-scale algal culture, Sci, Monthly, 80, 21-28.

Levich, V. G., 1962, Physiochemical Hydrodynamics, Prentice Hall, Inc., Englewood Cliffs, N. Y., 700 pp.

Lorenzen, H. 1963, Temperatureinflüsse auf Chlorella pyrenoidosa unter besonderer Berücksichtigung der Zellenwicklung, Flora, Jena, 153, 554-592.

Lucas, H. L., 1964, Stochastic elements in biological models; Their sources and significance, in Stochastic Models in Medicine and Biology, J. Gurland, ed., Univ. of Wisconsin, Madison, 383 pp.

Ludwig, C. A., 1938, The availability of different forms of nitrogen to a green alga (Chlorella). Am J. Bot., 25, 448-458.

Lüning, K and M. Neushul, 1978, Light and temperature demands for growth and reproduction of Laminarian gametophytes in southern and central California, Mar. Bio., 45, 297-309.

REFERENCES (CONT.)

Manahan, S. E. and M. J. Smith, 1973, Copper micronutrient requirement for algae, Env. Sci. Tech., 7, 829-833.

Margerum, D. W. and B. A. Zabin, 1962, Coordination kinetics by ion exchange, J. Phys. Chem., 66, 2214-2218.

McDuff, R. E. and F. M. M. Morel, 1973, Description and use of the chemical equilibrium program REDEQL2, Tech. Rpt. EQ-73-02, W. M. Keck Eng. Lab., California Inst. of Tech., Pasadena, CA, 82 pp.

Matsui, I., 1976, Catalytic oxidation of aqueous Mn(II), J. Catalysis, 41, 108-123.

Mölisch, H., 1895, Die Ernährung der Algen (Süsswasseralgen I. Abhandl), Sitzber, Akad. Wiss., Wien, Math. natur w. Kl. Abt. I., 104, 783-800.

Moody, J. R. and R. M. Lindstrom, 1977, Selection and cleaning of plastic containers for storage of trace element samples, Ana. Chem., 49, 2264-2267.

Morel, F. M. M., J. C. Westalls, J. G. Rueter and J. P. Chaplick, 1975, Description of the algal growth media "Aquil" and "Fraquil," R. M. Parsons Lab., Dept. of Civil Eng., Mass. Inst. Tech., Tech. Note No. 16, 33 pp.

Morel, F. M. M., J. G. Rueter, D. M. Anderson and R. R. L. Guillard, 1979, Aquil: A chemically defined phytoplankton culture medium for trace metal studies, J. Phycol., 15, 135-141.

Morgan, J. J., 1967, Chemical equilibria and kinetic properties of manganese in natural waters, in Principles and Applications of Water Chemistry, S. D. Faust and J. V. Hunter, eds., John Wiley and Sons, Inc., N. Y., 643 pp.

Müller, D., 1962, Über jahres and lunarperiodische Ersheinungen bein einigen Braunalgen, Bot. Marina, 4, 218-219.

Nancollas, G. H., 1966, Interactions in Electrolytic Solutions Elsevier Publ. Co., Amsterdam, N. Y., 214 pp.

North, W. J., 1971, The biology of giant kelp beds (Macrocystis)in California, Nova Hedwigia, 32, 1-660.

North, W. J., 1976, Marine Farm Project, September Progress Report, Keck Eng. Lab., California Institute of Technology, 111 pp.

REFERENCES (CONT.)

North, W. J., 1977a, Growth factors in the production of giant kelp, in Symposium on Clean Fuels from Biomass and Wastes, J. W. White and W. McGrew, eds., Inst. of Gas Technology, Orlando, FL, 521 pp.

1977b, Algal nutrition in the sea - some thoughts about management possibilities, in Symposium on Marine Plant Biomass of the Pacific Northwest Coast, Oregon State Univ., Corvallis, OR, 24 pp.

1977c, Marine Farm Studies, August Progress Report, Keck Eng. Lab., California Inst. of Tech., 104 pp.

North, W. J. and P. A. Wheeler, 1977, Nutritional requirements of Macrocystis, Ninth International Seaweed Symposium, Santa Barbara, CA, 11 pp.

North, W. J., 1978a, Progress in studies of oceanic production of biomass, Second Annual Symposium on Fuels from Biomass, Rensselaer Polytechnic Inst., Troy, N. Y., 38 pp.

1978b, Adverse factors affecting giant kelp and associated seaweeds, in Symposium on Pathogens of Marine Algae, Third International Congress of Plant Pathology, Technische Univ., Areisstrasse 21, Munich, Germany, 24 pp.

North, W. J., 1979, Quarterly and Monthly Progress Report, September, 1979, Marine Farm Project, California Inst. of Tech., 33 pp.

Parekh, J. M., J. Bhala, S. T. Talreja and Y. A. Doshi, 1969, Uptake of I^{131} by marine algae and the factors influencing iodine enrichment by seaweeds in seawater, Curr. Sci., 38, 268-269.

Pirson, A., H. Lorenzen and A. Koepper, 1959, A sensitive stage in synchronized cultures of Chlorella, Pl. Physiol., Lancaster, 34, 353-355.

Powls, R. J., J. Wong and N. I. Bishop, 1969, Electron transfer components of wild-type and photosynthetic mutant strains of Scenedesmus obliquus D_3, Biochem. biophys. Acta, 180, 490-499.

Prask, J. A. and D. J. Ploche, 1971, A role for zinc in the structural integrity of the cytoplasmic ribosomes of Euglena gracilis, Pl. Physiol., Lancaster, 48, 150-155.

REFERENCES (CONT.)

Price, C. A., 1968, Iron compounds and plant nutrition, Annual Rev. Pl. Physiol., 18, 239-248.

Provasoli, L. and J. J. Pintner, 1953, Ecological implications of in vitro nutritional requirements of algal flagellates, Ann. N. Y. Acad. Sci., 56, 839-851.

Provasoli, L., J. J. A. McLaughlin and M. R. Droop, 1957, The development of an artificial media for marine algae, Arch. Mikrobiol., 25, 392-428.

Provasoli, L., 1968, Media and the prospects for culturing marine algae, in Cultures and Collections of Algae, Proceedings of the U.S.-Japanese Conference, Hakone, Japan, A. Watanabe and A. Hattori, eds., 75 pp.

Reisner, G. S. and J. F. Thompson, 1956, Manganese deficiency on Chlorella under heterotrophic carbon nutrition, Nature, 178, 1473-1474.

Richards, F. A., 1965, Anoxic basins and fjords, in Chemical Oceanography, Vol. 1, Chap. 13, J. P. Riley and G. Skirrow, eds., Academic Press, N. Y., 712 pp.

Romheld, V. and H. Marschner, 1979, Effect of Fe-stress on utilization of iron chelates by efficient and inefficient plant species, in International Symposium on Trace Element Stress in Plants, W. L. Berry and A. Wallace, eds., Univ. of California, Los Angeles, CA, 73 pp.

Robinson, R. A. and R. H. Stokes, 1959, Electrolytic Solutions, Butterworths, London, 517 pp.

Scherfig, J., P. S. Dixon, R. Appleman and C. A. Justice, 1973, Effect of phosphorous removal processes on algal growth, EPA 660/3-73-015, Washington, D. C., 81 pp.

Scott, R. and L. E. Ericson, 1955, Some aspects of cobalt metabolism by Rhodymenia palmata with particular reference to vitamin B-12 content, J. Exptl. Bot., 6, 348-361.

Somer, I. I. and J. W. Shive, 1942, The iron-manganese relation in plant metabolism, Pl. Physiol., 17, 582-602.

Sorokin, C., 1959, Tabular comparative data for low-and high-temperature strains of Chlorella, Nature, London, 184, 613-614.

REFERENCES (CONT.)

Spencer, C. P., 1957, Utilization of trace elements by marine unicellular algae, J. Gen. Microbiol., 16, 282-285.

Steward, W. D. P., 1974, Algal Physiology and Biochemistry Univ. of California Press, Los Angeles, CA, 989 pp.

Strickland, J. D. H. and T. R. Parsons, 1972, A Practical Handbook of Seawater Analysis, Bulletin 167, Fisheries Research Board of Canada, Ottawa, 310 pp.

Stumm, W. and J. J. Morgan, 1970, Aquatic Chemistry, Wiley Interscience, N. Y., 583 pp.

Subramanian, K. S., C. L. Chakrabarti, J. E. Sueirasand and I. S. Maines, 1978, Preservation of some trace metals in samples of natural waters, Ana. Chem., 50, 444-448.

Sunda, W. G. and R. R. Guillard, 1976, The relationship between cupric ion activity and the toxicity of copper to phytoplankton, J. Mar. Res., 34, 511-529.

Sunda, W. G. and J. M. Lewis, 1978, Effect of complexation by natural organic ligands on the toxicity of copper to a unicellular alga, Monochrysis lutheri, Limnol. Oceanogr., 23, 870-876.

Taylor, W. R., 1963, Inorganic nutrient requirements for marine phytoplankton organisms, Exptl. Mar. Ecol., Narragansett Marine Lab., Univ. of R. I., 2, 17-24.

Teicher-Zallen, D., 1969, The effect of manganese on chloroplast structure and photosynthetic ability of Chlamydomonas reinhardi, Pl. Physiol., Lancaster, 44, 701-710.

Toerien, D. F., C. H. Huang, J. Radimsky, E. A. Pearson and J. Scherfig, 1971, Final Report, Provisional Algal Assay Procedures, EPA 1601-DQB, 211 pp.

Truesdell, A. H. and B. F. Jones, 1974, Wateq, A computer program for calculating chemical equilibrium of natural waters, J. Res. U. S. G. S., 12 (2), 233, 248.

Tsui, C., 1948, The role of zinc in auxin synthesis in the tomato plant, Am. J. Bot., 35, 172-179.

REFERENCES (CONT.)

Vuceta, J., 1976, Adsorption of Pb(II) and Cu(II) on α - Quartz from aqueous solutions: influence of pH, ionic strength, and complexing ligands, Ph. D. Thesis, California Inst. of Tech., Pasadena, CA, 206 pp.

Vuceta, J. and J. J. Morgan, 1978, Chemical modeling of trace metals in fresh waters: Role of complexation and adsorption, Env. Sci. Tech., 12, 1302-1309.

Walker, J. B., 1953, Inorganic micronutrient requirements of Chlorella, I. Requirements for calcium (or strontium) copper and molybdenum, Arch. Biochem. Biophys., 46, 1-11.

Walker, J. B., 1954, Inorganic micronutrient requirements of Chlorella II. Quantitative requirements for iron, manganese and zinc, Arch. Biochem. Biophys., 53, 1-8.

Wasserman, A., 1948, Cation adsorption by brown algae, the mode of occurrence of alginic acid, Ann. Bot., London, 13, 79-88.

Wheeler, W. N., 1978, Ecophysiological studies on the giant kelp, Macrocystis, Ph. D. Thesis, Biology Dept., Univ. of California, Santa Barbara, 179 pp.

Yamamoto, T., 1965, The cobalt and nickel contents of seaweeds, Nippon Kagaku Zasshi, 86, 53-59.