GROWTH, NUTRIENT UPTAKE AND CARBOHYDRATE PRODUCTION IN LABORATORY CULTURES OF SPIRULINA MAJOR (CYANOPHYCEAE)

F I N A L R E P O R T

by

C. F. Rhyne, Ph.D.

L. A. Crump

Jackson State University
Jackson, Mississippi 39217

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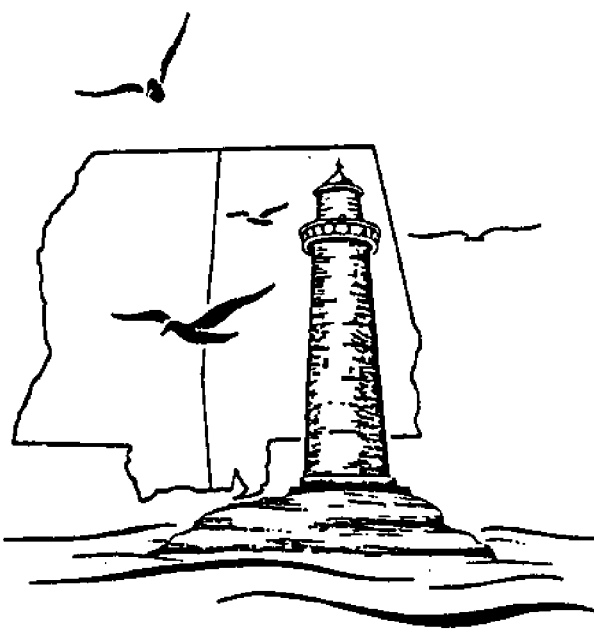

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Final Report

Prepared by:

C. F. Rhyne, Ph.D.
L. A. Crump
Jackson State University
Jackson, Mississippi

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This final report represents the work carried out under the 1981 project entitled, "Marine Algae in the Production of Fermentation Alcohol and in Wastewater Recovery", and the 1982 project entitled, "Marine Algae in the Production of Fuel/Chemical Feedstocks and in Wastwater Recovery".

This report also represents in its entirety the MS Thesis for L. Crump, under the same title, directed by C. F. Rhyne.

OVERALL OBJECTIVES

The basic aim of this study was to develop and analyze a photosynthetic bioconversion system utilizing marine algae as the basis for the production of a useable feedstock (algae food reserve) by achieving the following:

Analysis of various marine algae found along the Mississippi and Alabama coasts based on growth potential in a wastewater/seawater mixture.

Evaluation of procedures used in maximizing production of food reserves in the selected marine algae.

Study of the wastewater treatment relative to NO_3, NH_4 and PO_4 utilized by the algae biomass.

ABSTRACT

Spirulina major, a filamentous blue-green alga, has been shown under laboratory conditions to meet certain criteria necessary for its use as a potential biochemical feedstock in a wastewater/aquaculture system.

The adaptability of *S. major* to various percentages of wastewater/seawater mixtures enhances its use in estuarine environments. Optimal growth conditions for *S. major* in the laboratory have been demonstrated to be, a temperature of 30 C, low light intensity, salinity range of 4-30 ppt, and a pH range of 8.5-10.5. Cultures have been observed to remain relatively free of contamination if the pH is kept within the range and the wastewater is membrane filtered.

Biomass productions of 130 mg dry wt/liter/day of *S. major* were obtained. Carbohydrate content of cells increased from 16.4% in cells in exponential growth to 42.2% in cells subjected to low nitrogen conditions.

Under laboratory conditions, reductions of eutrophicating nutrients were found to be approximately 97% for NH_4, 100% for NO_3, and 47% for PO_4.

The attached growth habit of *S. major* allows for ease of harvest. Glass has been demonstrated to be a good substrate in providing additional surface area for growth and harvesting.

Additions of human urine (0.1-0.5%) under laboratory conditions appears as good as domestic/commercial wastewater as a useable source of nutrients to generate algal biomass.

INTRODUCTION

During the past several years, prices of petroleum products have risen sharply, a trend likely to continue, and one creating a serious problem for the chemical industry, which is heavily dependent upon non-renewable petrochemical feedstocks. To meet our chemical, energy, feed and food needs, alternative strategies capitalizing on renewable natural products are being investigated by various nations around the world.

The potential of carbohydrates as chemical feedstocks has been demonstrated but has not yet been significantly realized commercially except in a few instances. By way of chemical degradation, chemical synthesis and fermentation, carbohydrates yield a variety of petrochemical, as well as other products (Kahn and Forage 1980). Fermentation techniques based on aerobic and anaerobic microbial reactions with carbohydrates show considerable promise. There is a tendency to focus attention on production of chemicals such as ethanol, butanol, acetone and glycerol, particularly as these uses are already established and fermentation techniques developed.

Many large petrochemical companies now recognize that future growth depends in part on industry's ability to improve efficiency in the use of oil and gas feedstocks and to develop promising alternatives (Peterson 1980). A development planned for use by petrochemical companies in the near future involves conversion of biomass thru fermentation to useful

chemical products and gasification of municipal waste to raise steam for electricity generation. Four renewable-crop or plant waste categories are being studied by these companies: (1) fermentable sugars, (2) cellulose-rich products and wastes, (3) corn and (4) other crops from energy farms. According to Union Carbide, by the year 2000, chemical feedstocks from sources other than oil and gas will constitute 10-15% of the total (Wishart 1978).

Biological fixation of CO_2 into chemical products is the only known way of providing renewable organic compounds. Until chemists can emulate the ability of plants to capture and store carbon from the atmosphere, we may have to rely on resourceful plant systems. Photosynthesis is the only method for solar energy conversion presently practiced on a large scale. This biological process supplies all our food energy as well as fiber and wood. Further, reserves of fossil fuels on which we depend for most other energy requirements are themselves products of photosynthetic conversion of solar energy accumulated over geological time. Unfortunately, we now are faced with having to recognize these resources as finite.

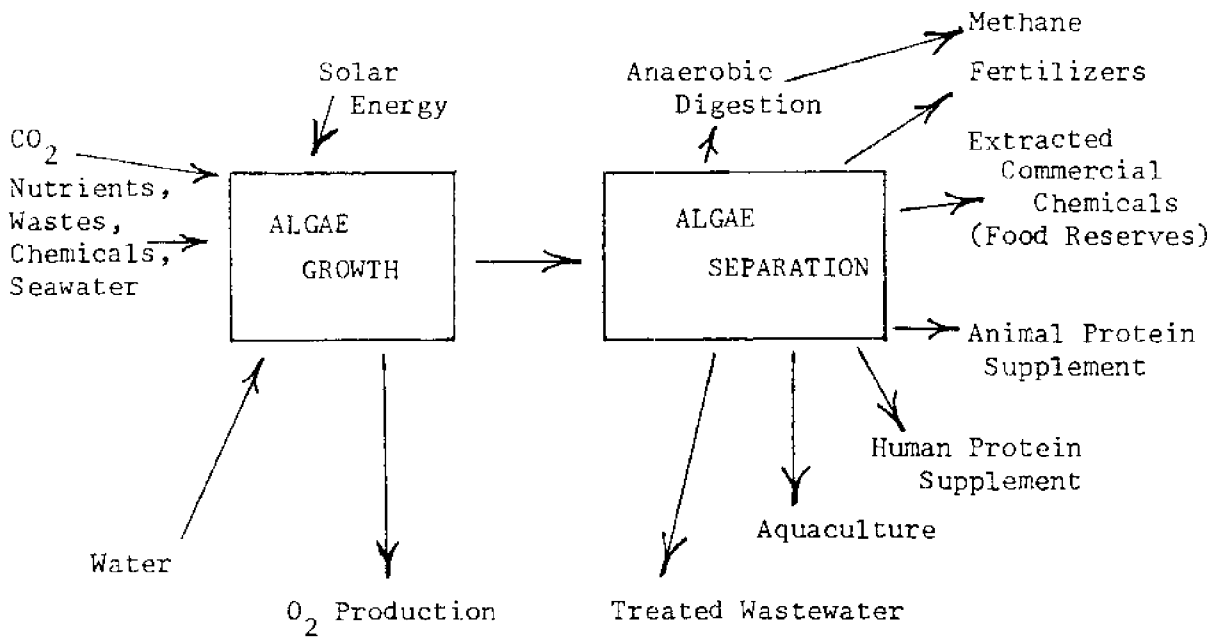
The amount of raw energy stored up in bioresources is simply staggering. In a single day the sun sends more energy to earth than could be consumed in an entire year. Although plants capture only one percent or less of that energy, they store some 20 times as much energy in a year as the world uses. Our most urgent task is to find out what the practical potential of biomass is for energy that humans can use, so that we can determine what the real problems and prospectus for bioenergy are (Peterson 1980).

Until recently, algae have mainly been considered as potential sources of fodder, food and biogas. Interest now is being focused on special algal species or growth conditions which permit the production of useful products such as fodder-protein simultaneously with useful basic chemical products such as glycerol, lipids or starch. These types of systems offer several opportunities for diversification, and could be of considerable interest in the context of gradual industrialization starting at the village level (Anderson et al. 1980, Lewis 1980, Wise 1980).

A great deal of interest has been generated recently in biomass-produced ethanol (Khan and Forage 1980, Brown 1980). Corn and certain other grain crops are proven excellent renewable sources of starch substrate appropriate for ethanol production, but the possibility of diverting invaluable food and international trade crops to long-term fuel production is receiving considerable opposition. Exploitation of the starch-substrate production potential of green plants that are not important food crops, and that may even be nuisance-species, would obviate objections based on risk to food supply and trade. Algae not only avoid the strictures placed on use of food crop plants, but are highly suitable as ethanol feedstocks.

In the past, mass culturing of algae has been done to produce single-cell protein (Burlew 1953, Tamiya 1957, Soeder 1980, Leone 1980). Recently, other potential practical applications of mass algae culture techniques have been advanced, including wastewater treatment, production of extractable chemicals and pharmaceutical botanicals, closed life-support systems, aquaculture and bioconversion of solar energy (Ryther

et al. 1972, Goldman and Ryther 1975, Benneman et al. 1977, Shelef 1979, Oswald and Benneman 1980, Jackson 1980). Starting with the basic photosynthetic reaction (and considering that NO_3 and PO_4 are essential nutrients for algae, in addition to the CO_2, water and light energy required for photosynthesis), it is possible to envision a multitude of potential production systems, such as that treated in the following figure (adapted from Goldman 1979).



One alga has met with popularity from the standpoint of harvest from natural sources and limited artificial culture. This is the blue-green alga Spirulina (Soeder 1980, Durand-Chastel 1980, Soong 1980, Vonshak et al. 1982, Nguyen et al. 1974, Nakamura 1970, Edwards 1980). The most popular of the Spirulina species are S. maxima (Mexican strain)

and S. platensis (African strain, Arthrospira platensis). These two species have received much recognition due to their nutritional quality and ease with which they can be harvested. Spirulina maxima is reported to have been part of the ancient Mayan culture as a food which was cooked in sauces and soups served over millet (Durand-Chastel 1980). Spirulina platensis is reported to be used by African tribes today (near Lake Chad) as a food which is harvested onto cloths and dried by the sun into cakes called "dihe" (Nakamura 1970).

The investigator proposed to study the production of a proven feedstock (cyanophycean starch) from an alga grown in seawater/wastewater mixtures. The study was designed to evaluate the growth potential, nutrient uptake of NH_4, NO_3 and PO_4 and carbohydrate production in the estuarine blue-green alga, Spirulina major grown in varying waste water/seawater combinations. The effects of wastewater and selected nutrient additions, temperature, salinity, substrate types and light energy variables were analyzed with optimization of biomass production and nutrient uptake in mind.

MATERIALS AND METHODS

EXPERIMENTAL ORGANISM

Spirulina major Kutzing (Smith 1950) is a filamentous multicellular blue-green alga. The trichomes of Spirulina major are helically twisted and are encased in a mucilaginous sheath that is uniform in density between trichomes. Thylakoids are few in number due to the small diameter of the alga and lie near the outer wall. The nucleoplasm lies near the inner wall and occupies the greater portion of each trichome. Under light microscopy, transverse walls appear to be obscure or lacking however, electron microscopy and staining has revealed that delicate transverse walls are present (Holmgren et al. 1971). Reproduction occurs by cell division. The storage product is a branched carbohydrate, glycogen, and alpha 1:4 linked glucan with 1:6 linkage (Trainor 1978). Species of Spirulina occur in both fresh and marine waters. Trichomes are actively motile, exhibiting both rotary and bending motions.

Classification of this organism according to Bold and Wynne (1978):

Division: Cyanochloronta

Class: Cyanophyceae

Order: Oscillatoriales

Family: Oscillatoriaceae

Genus: Spirulina

Species: major

A wild species of Spirulina was isolated from a water sample obtained from a culture tank located at the Oyster Laboratory of the Gulf Coast Research Laboratory in Biloxi, Mississippi. These tanks contained algae of all kinds with the predominant being Ulva lactuca, at the time of collection. The Spirulina in the water sample was separated by micro-separation and media preference.

Microseparation and Media Preference

This method was carried out with the use of micro needles made in the laboratory from Pasteur pipettes drawn into fine hollow points by heating in a flame. These then were attached to a vacuum pump system through which suction was applied. Spirulina filaments were drawn into the micropore of the needle and placed in seawater/Alga-gro (Carolina Biological Supply Co.) medium, pH 8.5. Cultures then were placed in an environmental chamber where the temperature was maintained at 30 C ± 1 C. A photoperiod of 15:9, L:D was maintained using 40 watt cool white fluorescent tubes.

STOCK CULTURE TECHNIQUES

Stock Water Supply

The stock seawater supply used in this study was collected in five gallon polyethylene carboys from a pump system at the Gulf Coast Research Laboratory, Oyster Laboratory located in Biloxi, Mississippi (10-20 ppt) and from Mississippi Sound, 15 miles off shore (30-35 ppt.). The wastewater effluent used was collected in carboys from the Municipal Wastewater Treatment Plant (trickling filter system) in Ocean Springs, Mississippi.

Chemical Analysis of Stock Water Supplies

Stock wastewater and seawater supplies were analyzed periodically to monitor the levels of NH_3-N, NO_3-N, PO_4 and Fe. Monitoring was carried out with the use of a reagent system (Bausch and Lomb Spectrokit) and a spectrophotometer (Bausch and Lomb Spectronic-20).

The Cadmium reduction method was used to assay the levels of nitrate present in the stock water supplies. This method required the application of reductant sulfanilic acid and amine coupling reagent. The reaction was allowed to proceed for 5 minutes, after which the sample was read at 540 nm.

The amount of ammonia present in the stock water supplies was determined by the nesslerization method. A dechlorinating reagent was added to the sample to remove chlorine. Nessler reagent was then added. After the reaction had proceeded for 10 minutes, the results were read at 410 nm.

Iron assays were done using the 1, 10-phenanthroline method which involves the chelation of one atom of ferrous iron by three molecules of 1,10-phenanthroline in an acetate buffered solution. Samples were read at 510 nm.

The phosphate test was carried out using the ascorbic acid method. Ammonium molybdate and potassium antimonyl tartrate react in an acid medium with dilute solutions of orthophosphate to form phosphomolybdic acid which is reduced to a molybdenum blue complex by ascorbic acid. This complex has an absorption maximum of 880 nm.

The pH of water stocks was monitored with a Fisher Accumet model 610 pH meter. Salinities were measured with an automatic temperature

compensated hand-held refractometer model 10419 (American Optical). Water was course filtered, then filtered through 0.45u membrane filters (Millipore) and stored in 5 liter Nalgene containers at 30 C.

Cultures

Stock cultures of Spirulina major were maintained in 1 and 2 liter Erlenmyer flasks in a medium composed of 24 ppt seawater that had been filtered through 0.45u filters. An artificial nutrient medium (Alga-Gro) Carolina Biological Supply Company was added in 20 ml amounts.

These stocks then were placed in a Percival reach-in environmental chamber model 160LL. Temperature was regulated to 30 C and monitored with a standard centigrade thermometer. Photoperiods of 15:9 L:D were provided with the use of 40 watt cool fluorescent tubes. Light energy was measured at 1.2 and 2.4 quanta/sec/cm^2 with the aid of a QSL-100 Quantum Scalar Irradiance meter. The pH of the stock medium was maintained between 8.5 and 10. Glass pipettes attached to rubber tubing and connect to a standard aquarium air pump (Willinger Bros.) provided aeration of the flasks. Approximately once a week, half of the liquid volume of each flask was removed and replenished with equal amounts of fresh medium.

EXPERIMENTAL PROCEDURES

Various parameters were used to determine which would provide optimum conditions for growth, viability and carbohydrate yield. The size of flasks (Erlenmyer, 250 and 500 ml) and the amount of medium (200 and 400 ml) added to each flask respectively were the only changes from the stock culture techniques.

Medium

Algo-Gro concentrate (Carolina Biological Supply Company) was used as a comparison medium. This enrichment was added to seawater of various salinities (6 to 35 ppt) and served as a control for each parameter tested.

Percentages of wastewater/seawater

Mixtures of wastewater/seawater were used in growth studies and were treated in the manner described previously.

Nutrient uptake

Samples of the medium were measured spectrophotometrically (Bausch and Lomb Spectrokits) for NH_4, NO_3 and PO_4 and iron.

Chemical additions

Measured amounts of the following compounds were added to a 50% wastewater/seawater mixture in individual flasks: $FeCl_2$, $Ca(NO_3)_2$, $NaCO_3$, $MgSO_4$ and K_2HPO_4. This was done to analyze their effect on the growth of the test organism.

Human urine from a healthy individual was used to analyze its effect on Spirulina. Urine was also used in conjunction with the above list of compounds.

Substrate tests

The following materials were used as a substrate to find out what effect increased surface area might have on the yield of Spirulina and to determine which substrate best served this purpose. The material used were measured strips of: "Ziplock" plastic bag; general polyethylene plastic; "Saran Wrap" plastic; cotton gauze and glass microscope slides.

Temperature studies

Temperature effects on the growth of Spirulina were carried out at two basic temperatures: 26 and 30 C.

Replication

All growth nutrient uptake and carbohydrate determinations were carried out using triplicate flasks unless otherwise indicated.

ANALYTICAL METHODS

A. Extraction of low-molecular weight components

Three samples of cells were collected and centrifuged to yield a packed volume of 0.5 ml. Supernatants were discarded and tubes containing the cells were placed on ice. Ten ml of ice cold 0.2 N $HClO_4$ were added to the cell pellets and vortexed to resuspend the cells. After 15 min at 4 C, samples were centrifuged in chilled centrifuge tube holders and the supernatant was carefully removed. This procedure was repeated with an additional 10 ml of perchloric acid.

B. Extraction of lipids

Ten ml of chloroform/methanol solution (1:1 v/v) was added to the pellets from the $HClO_4$ extraction and allowed to stand for 5 minutes at room temperature. Samples then were centrifuged and supernatants discarded. This procedure was repeated with 5 ml of chloroform-methanol.

C. Determination of proteins and carbohydrates

To the acid-extracted lipid-free pellets one ml of 1 N NaOH was added. The pellets were then placed in a boiling water bath for 10 minutes to dissolve the pellets.

Aliquots of the samples were assayed for carbohydrate by the phenol-sulfuric acid assay and for protein by the dye-binding assay.

Carbohydrate Determination

To determine the carbohydrate content of the samples the phenol-sulfuric acid method was used. This is a simple assay that is rapid, inexpensive and highly sensitive (Kochert 1980).

Materials

A. Reagents:

1. 90% phenol solution;

2. H_2SO_4 reagent grade (95.5%, specific gravity 1.48).

B. Solutions:

1. The phenol reagent was made by adding 10 ml of H_2O to 90 ml of the 90% phenol solution. The resultant solution is colorless, but may develop a pale yellow color in time. The color does not interfere with the assay, and the solution is usable for many months when stored at room temperature.

2. Glucose standard solution consists of 50 mg of glucose dissolved in water to a final volume of 100 ml.

Method

A standard curve was generated by pipetting a range (10-140 l) of glucose standard solution into a series of marked 18 x 150-mm test tubes. Volume was adjusted to 2 ml with water in each tube. A reagent blank was included containing 2 ml of water only. To these tubes, 50u l of phenol reagent was added and mixed thoroughly. Five ml of H_2SO_4 was rapidly added to each tube, directing it to the surface of the liquid in the tube, with a 5 ml pipette that had a portion of the tip removed to facilitate rapid dispensing. Total delivery time should be 15-20 sec for 5 ml. The samples then were allowed to stand at room

temperature for 30 min as the procedure promotes heat and mixing that is necessary for the assay. The weight of glucose standard was plotted against the absorbance to generate a curve.

The unknown carbohydrate samples were pipetted into separate marked test tubes in different volumes (three-four), and their volumes were adjusted to 2 ml with the buffer (NaOH). These samples then were treated with the reagents and allowed to cool at room temperature for 30 min. The absorbance was read at 485 nm on a (Spectronic-20, Bausch and Lomb) spectrophotometer. Each unknown carbohydrate concentration was determined graphically.

RESULTS

The yields of Spirulina major appear to be somewhat higher than that of Spirulina sp. after both a five and eight day growth study (Tables 1-2). During the eight day test the 40-60% wastewater flasks showed the greatest yields for both species. Controls of Alga-gro medium, however, did produce the greatest growth (Table 2). This was not the case in the five day study (Table 1).

Spirulina major and S. sp. showed somewhat similar yields with additions of various nutrient supplements (Table 3). Ferrous chloride appeared to raise the yield for both species over the other compound additions. The "all chemicals" addition did, however, show the best yield for both species.

When a suitable substrate (polyethylene strip) was used in conjunction with iron additions, the yield increased considerably over those tests without substrate (Table 4). Table 5 shows that the cotton gauze strip produced a higher yield per unit area than the remaining substrate types. When substrates of plastic and aluminum versus no substrates were tested at 35 C, the yields for the no substrate flasks were significantly higher than those with plastic substrate, but not significantly higher than the aluminum substrate (Table 6). Tests at 30 C in urine/seawater media showed significantly lower yields for aluminum screening compared to no substrate at all. The yield at 30 C for no substrate was however, higher than at 35 C (Table 6). Table 7 shows that glass

substrates accounted for higher yields than polyester meshing in all nutrient combinations but one.

The results of various additions on the growth of S. major using unfiltered wastewater, urine, seawater and inorganic compounds show that three combinations tended to enhance yield over the remaining tests. These being: (1) seawater plus 0.1% urine and iron; (2) seawater plus four inorganic supplements and (3) 60% wastewater/seawater plus iron (Table 8).

Table 9 shows that single additions of $FeCl_2$, $FeCl_3$ and K_2HPO_4 added to 0.1% urine/seawater mixtures enhanced yield over that of other compound additions.

When refrigerated versus fresh catch urine were compared for their effectiveness, the refrigerated samples showed at least as good or better growth enhancing qualities (Table 10).

Table 11 shows the significant yield differences between the two test temperatures of 26 and 30 C and light energy levels of 1.6, 2.4, and $4.0 \cdot 10^{16}$ quanta. cm^2. sec^{-1}.

The reduction of the three plant nutrients NH_4, NO_3 and PO_4 are seen in Table 12. Virtually all of the NO_3 and NH_4 is removed by both the Spirulina major and S. sp. culturing systems. Spirulina major removed 47% of the PO_4 while S. sp. removed 62% of the PO_4 in 8 days.

Carbohydrate analysis of Spirulina major revealed that in all cases, carbohydrate content of the senescent cells was higher as compared to exponential cells. Table 13 shows replicate carbohydrate assay values in a control medium of Alga-Gro. The senescent cells at each sampling were high in carbohydrate ($\bar{x}$:38%) as compared to exponential cells ($\bar{x}$:25.8%).

Further analysis of cells grown in wastewater/seawater mixtures (Tables 14-15) demonstrated again that senescent cells have higher carbohydrate levels (38.5%, 40.5% and 42.2%) as compared to exponential cells (16.4%).

Table 1. Yield of *Spirulina major* and *Spirulina* species in Wastewater/Seawater Mixtures

Medium	*Spirulina major** mg/liter dry wt.	*Spirulina* sp.* mg/liter dry wt.
Control**	615.5	217.9
10% Wastewater	654.5	542
20% Wastewater	623.5	590.5
40% Wastewater	620	351.2

*8 day growth period
Temperature: 26°C
**Alga-Gro Medium

Table 2. Yield of *Spirulina major* and *Spirulina* species in Wastewater/Seawater Mixtures

Medium	*Spirulina major* mg/liter dry wt.	*Spirulina* sp. mg/liter dry wt.
Control*	191.8	158.5
40%**	109	106
40%***	128	144.5
60%***	133.5	120.5
80%***	81.5	96
100%	38.5	16

*in 17 ppt and Alga-Gro (nutrient enrichment)
**in 14 ppt seawater
***in 17 ppt seawater
growth period of 5 days
Temperature: 26°C

Table 3. Yield of *Spirulina major* vs. *Spirulina* species

Medium Additions*	*Spirulina major* mg/liter dry wt.	*Spirulina* sp. mg/liter dry wt.
$FeCl_2$ (0.8 mg/l)	31	37.5
$Ca(NO_3)_2$ (40 mg/l)	18	17.5
$NaCO_3$ (20 mg/l)	23.5	20
$MgSO_4$ (25 mg/l)	22	22.5
K_2HPO_4 (100 mg/l)	23	18
All Chemicals	99.5	48

*Chemicals added to a 50% wastewater/seawater mix
Growth period - 6 days

Table 4. Growth Experiment-*Spirulina major*

Medium*	Biomass mg/l dry wt.***
Control	119.85
Control plus 0.1 ml $FeCl_2$ (0.4 mg/l)	183.70
Control plus 1.0 ml $FeCl_2$ (4 mg/l)	201.35
Control plus 1.0 ml $FeCl_2$ (4 mg/l) and (5"x2") polyethylene strip**	287.35

*Medium 60% wastewater/seawater and was changed every third day.
**Area of strip 64.52 cm^2.
***7 day growth period

Table 5. Substrate and Growth Study of *Spirulina major*

Medium* Substrate	BIOMASS (mg/l dry wt./substrate)*
Strip of "Ziploc" bag**	48.70 (0.54 mg/cm^2)
Strip of polyethylene** shipping bag	68.0 (0.76 mg/cm^2)
Strip of cotton gauze**	137.20 (1.52 mg/cm^2)
Strip of "Saran" wrap**	111.60 (1.23 mg/cm^2)
Inside surface of glass*** tank	314.10 (0.24 mg/cm^2)

*Medium 50% mix of wastewater/seawater with 4 mg/l $FeCl_2$
**Area of strips 90.32 cm^2
***Area of inside surface of culture tank 1292 cm^2.
5 day growth period
Values in parentheses indicate the weight of biomass per unit area of substrate.

Table 6. Growth of Spirulina major in Relation to Substrate and Medium Conditions

Medium	Substrate	BIOMASS mg/l dry wt.*
60% wastewater/seawater plus 4 mg/l $FeCl_3$ 35 C	plastic screening	150
	Aluminum screening	180
	no screening	185
Seawater plus 0.5% human urine and 4 mg/l $FeCl_3$ 35 C	plastic screening	155
	Aluminum screening	330
	no screening	360
Seawater plus 0.5% human urine and 4 mg/l $FeCl_3$ 30 C	Aluminum screening	288
	no screening	442

Yield after 5 day growth period

Table 7. Nutrient Enhancement and Substrate Study of Spirulina major

Medium	Substrate	BIOMASS mg/l dry wt.
Control-1* seawater with H_3BO_3 (2.45 mg/l)	small mesh	235
	large mesh	185
	microslide**	295
Control-2* 60% wastewater/seawater	small mesh	280
	large mesh	360
	microslide	280
60% wastewater/seawater with $MnSO_4$ (125 mg/l)	small mesh	145
	large mesh	130
	microslide	310
60% wastewater/seawater with $FeCl_2$ (4 ml/l)	small mesh	235
	large mesh	160
	microslide	260
60% wastewater/seawater with $FeCl_3$ (4 mg/l)	small mesh	210
	large mesh	235
	microslide	335

NOTE: Mesh substrates are of polyester cloth fiber.
**Glass slide (25mm x 75mm)

Table 8. Nutrient Enhancement of Spirulina major

	Medium	Biomass mg/l dry wt.*
1.	Seawater 0.1% human urine plus 4.0 mg.l FeCl	220
2.	Seawater plus $MgSO_4$, $CaCO_3$, $FeCl_2$, K_2HPO_4 (125 mg/l, 100 mg/l, 4 mg/l, 500 mg/l)	225
3.	Seawater plus 0.5% human urine and $FeCl_2$ (4 mg/l)	125
4.	10 ml unfiltered wastewater in seawater plus 0.1% human urine	70
5.	50% wastewater/seawater plus $FeCl_2$ (4 mg/l)	235
6.	Seawater plus 0.5% human urine with additions of chemicals as in #2	135
7.	10 ml of unfiltered wastewater in seawater plus 0.5% human urine	70
8.	60% wastewater/seawater mixed with additions of chemicals in #2 plus 0.5% human urine	135
9.	10 ml unfiltered wastewater in seawater with 0.1% human urine	100
10.	10 ml of unfiltered wastewater in seawater plus 1% human urine and $FeCl_2$ (4 mg/l)	125

*Yield after five day growth period.

Table 9. Effects of Nutrient Additives on Growth of Spirulina major

Medium Addition	Biomass mg/l dry wt.
$Ca(NO_3)_2$ (100 mg/l)	65
$FeCl_2$ (2 mg/l)	135
K_2HPO_4 (250 mg/l)	70
$MgSO_4$ (62.5 mg/l)	75
$Ca(NO_3)_2$ (1000 mg/l)	75
$FeCl_2$ (20 mg/l)	125
K_2HPO_4 (2500 mg/l)	175
All chemicals above in seawater (less human urine)	70
All chemicals above in seawater	100
60% wastewater/seawater mix (less human urine)	75

*All medium composed of seawater---plus 0.1% human urine except where indicated.

Yield after four day growth period.

Table 10. Salinity and Nutrient Study of *Spirulina major*

Medium	Biomass mg/l dry wt.
* Seawater 25 ppt. plus 0.5% human urine and $FeCl_3$***	19
* Seawater 10 ppt. plus 0.5% human urine and	19
**Seawater 25 ppt. plus 0.5% human urine and	16
**Seawater 10 ppt. plus 0.5% human urine and	14

*Refrigerated human urine.
**Fresh catch human urine.
***Concentration $FeCl_3$ (1.6 mg/l)
Five day growth period.

Table 11. Effects of Temperature and Light Energy on Growth of Spirulina major

Medium (Five day experiment)	BIOMASS (mg/l dry wt.) TEMPERATURES** 26 C	30 C
Flask A***	30	215
Flask B	38	206.5
Flask C	39.5	215.75
Flask D	54	112.75

* The basic medium consisted of 50% wastewater/seawater mix. Additions of 4 mg/l $FeCl_2$ in Flasks A,B.C.D. (125 mg/l) $MgSO_4$ and (50 mg/l) K_2HPO_4 added to Flask D only.

**Irradiance measured in environmental chambers:
26°C--2.4 x 10^{16} quanta/cm^2/sec^{-1}
30°C--4.0 x 10^{16} quanta/cm^2/sec^{-1}

***Flask A 26°C Irradiance: 1.6 10^{16} q/cm^2/sec^{-1}
Flask A in 30° E.C., Irradiance: 2.4 10^{16} q/cm^2/sec^{-1}

Table 12. Reduction of Ammonia, Nitrate and Phosphate by Spirulina major and Spirulina sp.

	Spirulina major			Spirulina sp.		
	NH_4	NO_3	PO_4	NH_4	NO_3	PO_4
Medium*	Percent reduction			Percent reduction		
$FeCl_3$	96.8	100	72.4	96.6	100	68.4
K_2HPO_4	96.5	100	35.9	96.6	100	51.7
$NaCO_3$	96.9	100	16.1	96.6	100	64.3
$MgSO_4$	98.1	100	56.6	95.4	100	62.6
$Ca(NO_3)_2$***	97	96.7	56.5	98.3	100	64.3
$\bar{x}$	97	99.4	47.5	96.7	100	62.2

* Chemical analysis of wastewater at beginning of test: NH_4: 14.5 mg l^{-1}, NO_3: 64.1 mg l^{-1}, PO_4: 4.6 mg l^{-1}

** Total PO_4: 6.4 mg l^{-1}

***Total NO_3: 94.3 mg l^{-1}

Eight day growth period

Table 13. Carbohydrate Analysis of Spirulina major

	Percent Carbohydrate	
	Exponential* cells	Senescent* cells
	7.10	19.7
	26.8	40.9
	26.8	45.8
	31.0	46.5
$\bar{x}$	25.8	38.0

*10 day growth period. Medium 30 ppt seawater and Alga-Gro.

Table 14. Carbohydrate Analysis of Spirulina major

Percent Carbohydrate	
Exponential* Cells	Senescent* Cells
9.9	36.0
22.9	45.1
16.4 ($\bar{x}$)	40.5 ($\bar{x}$)

*An average of three samplings. Growth medium 60% wastewater/seawater mix. +4 mg/l $FeCl_2$.

Exponential cells - 3 days old

Senescent cells - 7 days old

Table 15. Carbohydrate Analysis of Senescent *Spirulina major* cells

Percent Carbohydrate Source of Cell Sample		
Stock*	30 ppt**	50% mix***
31.7	26.1	35.3
45.8	56.4	51.1
38.8 ($\bar{x}$)	41.3 ($\bar{x}$)	42.2($\bar{x}$)

* 60% wastewater/seawater mix. 20 day growth period.
** Alga-Gro in 30 ppt seawater, 12 day growth period.
***50% wastewater/seawater mix + 4 mg/l $FeCl_2$, 22 day growth period.

DISCUSSION

Heretofore, microalgae cultures were maintained for their high protein producing qualities (Nakamura 1970, Waslein et al. 1978, Hills 1980). In terms of protein production per unit area, microalgae are more efficient than any other type of plant (Waslien et al. 1978).

Within recent years, the need for renewable fuel and chemical feedstocks prompted the investigation of alternative energy systems. One of these being high rate algal ponds based on wastewater effluent and solar energy.

Oswald (1980) had demonstrated algal growth as being a highly economical method of advanced wastewater treatment and the most efficient known way to fix solar energy in the form of biomass. The potential of algae to provide abundant food, feed and energy has raised questions concerning harvest methods, predator infestations, contamination, species selection and economy of scale. Bennemenn (1980) states that wastewater treatment with a potential for net energy production, rather than consumption in the form of wastewater aquaculture can be achieved at low cost.

There are certain criteria that have to be met in order to realize effective wastewater/mariculture systems. The major requirements are: (1) moderate to high productivity, (2) adaptability to high wastewater levels in seawater, (3) reasonable resistence to contaminating algae, (4) high levels of plant nutrient uptake, (5) be easily harvested,

(6) relatively high production of a useable feedstock (protein, pigment, glycerol etc.).

Each of these areas will be discussed in light of the data collected.

Our observations of Spirulina major in wastewater/seawater mixtures under laboratory conditions, demonstrate its potential as a biochemical feedstock. In a comparative study of Spirulina major and a wild Spirulina species obtained from a water sample from the Oyster Laboratory in Biloxi, Mississippi, our findings show that S. major on the average provided greater biomass yields. The adaptability of S. major to various percentages of wastewater/seawater mixtures enhances its use in estuarine/wastewater environments.

Holmgren et al. (1971) are some of the very few investigators to have studied S. major. Their work consisted of ultrastructure studies of crosswalls in S. major and their relationship to another blue-green genus Arthrospira.

The ability of S. major to adapt to various seawater salinties is remarkable. Our observations show that it grew in salinities ranging from 4 to 30 ppt. Reports by Goldman (1980) indicate that several species of Spirulina have wide tolerances to salinity and could grow in supplemented seawater under laboratory conditions.

Few if any studies have been made of artificial substrates as possible means of increasing growing area and facilitating harvesting and reseeding of blue-green algae. Most other species of Spirulina are planktonic. Additional substrates within growth flasks provided extra space for increased yield of S. major over controls.

Cotton gauze strips as substrates increased yield but tended to disintegrate after 24 hours. It appeared that the pH and perhaps some

enzymatic activity caused the gauze to breakdown. Aluminum screening showed inconclusive results in that yields were both above and considerably below controls in all tests. Polyester mesh did not demonstrate as good a yield as glass; however, we feel that further tests may prove this material superior in both yield and reseeding qualities. Glass slides proved to be the best substrate in consistently producing larger yields.

Total carbohydrate as predominately the storage product, glycogen, was greater in senescent cells of S. major averaging approximately 21% in actively growing cells versus 40% in senescent cells. Nutrient levels in the medium of senescent cell cultures were found to be nearing depletion. Nitrogen was the limiting factor in this case. When nitrogen concentrations were high, cell assays indicated high protein and low carbohydrate levels. Bennemann (1980) reported that when nitrogen was the limiting factor, glycogen increased 400% in Spirulina. Nguyen et al. 1974 reports that Spirulina grown in wastewater effluent increased its carbohydrate production because of the effluent's very low nitrogen content.

Both S. major and S. sp. demonstrated very high rates of NH_4 and NO_3 reduction. Both species reduced NH_4 by approximately 97% and NO_3 by 100%. Reductions of PO_4 were found to be about 47% in S. major and 62% in S. sp.

We have used a waste product not normally seriously considered by many at least in this country to be a important component in an algal growth medium. However, when thinking of wastewater aquaculture, human urine may well play an important role in enhancing productivity

of certain algae species. It has been shown that urine from a healthy individual is basically sterile and should represent a nutrient addition considerably "cleaner" than secondarily treated domestic and commercial wastewater.

Almost 100 nitrogenous compounds are found in human urine and range in concentration from traces to approximately 24 g of urea expelled by the normal healthy adult each day (Altman and Dittmer 1968). Depending on the microbial flora in the receiving waters, these nitrogenous compounds will impart a high NH_4 level. A 0.5% addition of fresh human urine to seawater raised the NH_4-N level by approximately 14 mg l^{-1} on an average during our studies. It appears that S. major had adapted to a level of 0.1-0.5% urine (1-5 ml·l^{-1}) in seawater. Urine also contains electrolytes, vitamins and related compound, lipids and carbohydrates, hormones, enzymes and miscellaneous organic compounds (Altman and Dittmer 1968). All of these elements and compounds undoubtedly act as either antagonists or promoters of algal growth at varying concentrations. Others have used Spirulina maxima (a Mexican strain) and S. platensis (an Ethiopian strain) in their investigations (Nguyen et al. 1974, Durand-Chastel 1980, Soong 1980). A temperature of 30 C and low-light energy ($1.6\text{-}2.4 \cdot 10^{16}$ quanta/cm^2/sec^{-1}) provide optimal growth conditions for S. major in our study. This is corroborated by others who have studied other Spirulina species (Nakamura 1970, Nguyen et al. 1974, Becker et al. 1980, Richmond et al. 1980, Payer et al. 1980, Soong 1980).

Becker and Venkataraman (1980) report that a limitation of growth of Spirulina occurred at high light intensities necessitating shading

of the cultures in large open ponds. Optimal growth of the cultures also required a temperature range of 25-30 C.

A pH range of 8.5-10.5 increased the growth of S. major and rendered cultures relatively free of contamination. When wastewater had not been membrane filtered, we observed considerable contamination by protozoans and assorted algal forms. Soong (1980) states that Spirulina grows well at high pH values, with the optimum for growth being between 8.5 and 9.5. If the pH was above 10.5 or below 7.0, growth was reduced. Similar results were found by Payer (1980) while studying pH levels relevant to growth of S. maxima.

Productivity of S. major increased in response to certain chemical additions. Best results were demonstrated when small additions of K_2HPO_4 and iron were made. Later testing of $FeCl_3$ showed that this single compound raised the yield over that of other nutrient compound additions.

Gordon (1979) reported that use of iron supplements in cultures of the green alga, Scenedesmus enhanced yield significantly. He further adds that although secondarily treated wastewater has low levels of iron, it contains adequate concentrations of nutrients to support substantial algae growth. Benneman (1980) noted an increase in the initial carbohydrate content in Scenedesmus when iron was the limiting factor in the medium.

Urine appears in our studies to do as well as domestic/commercial wastewater as a useable source of nutrients in generating algal biomass in controlled laboratory systems. Urine from healthy individuals for the most part does not carry the dangers of pathogens, heavy metals and toxic organic compounds sometimes found in secondarily treated wastewaters.

PROJECT SUMMARY

Analysis of data generated by the laboratory oriented culture system demonstrated that *Spirulina major* could possibly meet the criteria needed for a successful wastewater/aquaculture system.

Adaptability to wastewater/seawater concentrations: *Spirulina major* was shown to grow well at levels up to 60% wastewater in seawater. Human urine at levels ranging from 0.1 to 0.5% appeared to support good growth. Iron as $FeCl_2$ and $FeCl_3$ was needed in both wastewater and urine based media for continued high yields.

Biomass Production: Up to 130 mg/l/day dry weight was observed (equivalent to 43 g/m^2/day in a system 0.33 m deep).

Carbohydrate Production: Yields up to 42% of dry weight were found in senescent cells.

Harvesting: Attached growth habit, easily removed from a smooth substrate.

Resistance to Contamination: Unialgal purity as long as pH is held between 9 and 10.

Nutrient Uptake: After eight day experiments, 100% of both the NH_4 and NO_3 and 47% of the PO_4 were calculated to have left the culture system by both algal activity and denitrification.

RECOMMENDATIONS

The attached forms of Spirulina, such as S. major, do show potential in the combined efforts of ameliorating plant nutrient levels in wastewater/seawater mixtures and producing crude feedstock products. However, a large scale field operation consisting of mixing seawater and wastewater would not be a feasible approach in raising Spirulina for production of feedstocks. To date, the daily variability of essential nutrients such as nitrate and phosphate in municipal wastewaters is large which helps create unstable growing conditions.

Large open growing areas such as one acre ponds are highly susceptible to contamination by both foreign algal species and herbivores. In order to help keep these open areas more conducive to monoculture of Spirulina, considerable additions of alkaline compounds such as $NaHCO_3$ would be needed to maintain relatively high pH conditions. Heavy rainfall will also change the salinity and dilute nutrients in unprotected pond areas helping to create further instability.

The investigator feels that the production of S. major will have to be increased substantially from the present 130 mg/l/day level to at least 0.5-1.0 g/l/day before serious thought can be given to scale up of this system. Increased biomass production may have to come from high growth rate strains of Spirulina either found naturally or bioengineered. Smaller aquaculture systems, perhaps 20-50,000 l/day based on seawater (10-25%) with additions of not wastewater but urine may help answer questions concerning wastewater generated algal biomass.

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