1974

The effect of sewage effluents and their constituents upon the vegetative growth of Ulva lactuca Linnaeus 1753 (Sea-lettuce) : progress report

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THE EFFECT OF SEWAGE EFFLUENTS AND THEIR CONSTITUENTS UPON THE VEGETATIVE GROWTH OF ULVA LACTUCA LINNAEUS 1753 (SEA-LETTUCE)

Progress Report

Submitted to

Hampton Roads Sanitation District Commission

by

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Gloucester Point, Virginia

March 1974

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I. Preliminary Statements

Ulva lactuca Linnaeus 1753, referred to in the vernacular as "Sea Lettuce" is a green, marine alga often forming large, distromatic blades of considerable size. It usually begins its existence from a single celled, germinating zygote or germinating zoospore. During its early stages it is almost always attached to some object. As it increases in size, it may become detached either by its sheer bulk, or by the action of rough waters. Once it is detached, however, it can continue its growth, often at a very rapid rate, if the environmental conditions are satisfactory. Large pieces may become fragmented through wave and beach interactions and by foraging and grazing animals. These fragments may also continue to grow as long as the conditions for such vegetative growth approach optimal.

Among these important environmental factors one may mention: 1) the photoperiod, 2) the light intensity, 3) the salinity or the brackishness of the water, 4) the temperature, and 5) the amount and type of available nutrients.

When the first four of these enumerated factors are approaching optimal, the fourth factor of nutrient

type and amount, when satisfactory, will cause seemingly almost unlimited growth. This condition is indeed encountered, especially during the summer months when warm waters prevail, about those municipalities which dump large and continuous amounts of sewage effluents into the marine waters. The resulting growth of this plant under these optimal conditions is often sufficiently great enough to cause nuisances. This growth can become especially critical in local areas where the sewage effluents are not adequately dispersed into the open waters of the ocean. Among such local areas are slow moving rivers, protected bays, and other impoundments with restricted access to open waters (Letts, 1908; Cotton, 1910).

This growth may be so profuse as to be a nuisance to bathing beaches and to residential areas located near the shore (Letts and Adney, 1908; Sawyer, 1965). In addition to making many beaches unfit for human activity by the sheer bulk of this rotting marine algal vegetation that has been cast ashore, there also occurs, simultaneously with this decay, the release of large amounts of hydrogen sulphide. This, in addition to being obnoxious to the olfactory senses, is also responsible for corrosion of metal surfaces and tarnishing of paints,

especially those with a white lead base (Cotton, 1910).

The Hampton Roads Sanitation District Commission and the Virginia State Water Control Board (Pollution Abatement Division) recognized that this is a potentially serious problem in the Elizabeth River in the Norfolk-Hampton Roads area. Mr. Millard Robbins, Director, Pollution Abatement Division, consulted Dr. Clair N. Sawyer, an investigator who had studied a similar problem in the Boston, Massachusetts area, an area where this had been a very serious problem (Sawyer, 1965).

Dr. Sawyer recommended that experiments be undertaken to establish the effect of various sewage effluents upon the growth of this plant, in particular, the effect of 1% levels of 1) raw sewage, 2) settled sewage, 3) unnitrified sewage, and 4) nitrified sewage.

The algae-culture laboratory at the Virginia Institute of Marine Science, Gloucester Point, Virginia, was engaged through Dr. Michael Bender to construct and execute experiments to establish the effect of those effluents on the vegetative growth of U. lactuca. It was also requested that experiments be undertaken to establish, if possible, whether or not the growth promoting substances could be identified. This type of information, subsequently, could hopefully be utilized

in formulating plans for the disposal of sewage effluents, and, in formulating possible treatment protocol for the removal of the growth promoting substances in the sewage treatment plant.

Before these main objectives could be realized, it was necessary to first establish the optimal parameters of vegetative growth in U. lactuca. A search of the literature revealed that little work had been done with the vegetative multiplication of U. lactuca, though a substantial amount of work had been published on zoospore and zygote development for the first two weeks after germination.

Of the essential parameters of vegetative growth in U. lactuca only the one of photoperiod had been investigated and published. All other parameters of vegetative growth had to be established in this laboratory before the actual test on sewage effluents and their derivatives could be undertaken.

II. Field Observations

The following general facts based upon observations made in the field played a roll in the choice and constructions of experiments:

1) Luxuriant growths of U. lactuca often occur in areas polluted by sewage, especially so in those areas where there is not free access to open waters, and, hence, a build-up of growth promoting pollutants may occur.
2) Since many municipalities are located on estuaries, the brackishness of the water may also be a contributing factor to the development of dense populations of U. lactuca.
3) The development of obnoxious populations occurs during the summer when water surface temperatures are usually in excess of 20°C, when light intensity is greatest, and when light duration is longest.
4) In order for U. lactuca to realize luxuriant growth it must have light. Therefore, for the most part, luxuriant growth of U. lactuca must occur where the water is sufficiently shallow, or transparent, or both to permit penetration to the bottom of enough light to support growth. This is especially true in U. lactuca as this is a benthic, attached alga. Detached plants or thalli of U. lactuca will also lie on the bottom except when disturbed by storms and currents.

III. Material

1. Source

Ulva lactuca Linnaeus 1753 was collected in the Elizabeth River on June 8, 1972, approximately one half mile south of the Norfolk naval yard. Only dark green blades were collected about the size of a football with natural, untorn margins. This was done to assure that flat bladed material was *U. lactuca* and not that of some wide bladed *Enteromorpha* such as *E. linza*.

2. Preparation for Isolation

A crisp, dark green, healthy blade was wiped clean on both sides with cotton balls until microscopical examination revealed that the surface was free of epiphytic algae. From this cleaned *Ulva* blade, a small section ca. 1 x 3 cm was cut. This cut out piece was chopped into very small fragments with a single edged razor blade. These small fragments were then spread over unenriched 1.5% agar-sea water plates. The surface moisture of the agar-sea water plates was allowed to evaporate.

3. Isolation

Isolation into unialgal cultures was achieved by picking up singly by use of drawn out, sterile, Pasteur pipettes pieces of this finely chopped material. One, and only one, fragment was placed individually into test tubes containing 15 ml of sterile Erdschreiber Enrichment, (see Appendix I for its preparation).

These isolated fragments were then incubated in a plant growth chamber, or phytotron, at 20°C at 250 ft c, under 16 hrs light-8 hrs darkness photoperiod. After three weeks there, growing fragments were examined microscopically for algal and fungal contamination. All subsequent investigations which follow were carried out on material derived from one of these growing fragments which proved to be free from contamination.

4. Maintenance and Multiplication of Isolate

The non-contaminated fragment on reaching ca. one centimeter in length was cut into four equal pieces with a sterile single edged razor blade. Each of these pieces was placed singly and individually into four 250 ml flasks containing ca. 125 ml of Erdschreiber Enrichment. When these isolated fragments reached ca. three to four square centimeters in size they were transferred to 2800 ml

Fernbach flasks containing two liters of N_2M medium. This medium is a high salinity sea water, collected at Wachapreague, Virginia, which has been enriched with nitrates, phosphates, trace elements, and vitamins (see Appendix II for its preparation).

The inoculated Fernbach flasks were placed under vigorous aeration with atmospheric air delivered by means of coarse fritted, pyrex glass, dispersion tubes. The air prior to passing through the cultures was washed in sterile distilled water and passed through sterile cotton plugs contained in 100 mm long, straight, bulbus calcium chloride drying tubes. The light intensity was 250-300 ft-c and continuous, i.e. there was no photoperiod. By these methods the pieces would double in size every three to four days. This growth rate is comparable to observations of growth rates made at Woods Hole on natural populations in non-polluted areas (Kanwisher, 1966). When these pieces reached ca. 100 cm^2, they were torn into two equal pieces. One of these pieces was placed into two liters of fresh medium and allowed to continue growth. The other piece was either used as inocula for the experiments described below, or, when not needed for these purposes, they were thrown away.

5. Genetic and Physiological Uniformity of Cultured Material

It should be emphasized here that all material hereby produced has, thus, been derived from a single growing fragment by vegetative divisions, and, hence, is genetically uniform. Since these genetically uniform pieces were grown under identical environmental conditions throughout the duration of the experiments to follow, they should be, for practical purposes, physiologically identical.

IV. Equipment

Plant Growth Chamber or Phytotron

Plant growth chambers or, perhaps more properly, phytotrons, manufactured by the Percival Refrigeration and Manufacturing Company, Boone, Iowa, (Percival No. I-60LLVL) were utilized in these investigations. These measured 55 3/4" by 28" by 52 3/4", or ca. 25 cu ft. These phytotrons permitted the manipulation of the environmental factors of 1) photoperiod, 2) temperature, and 3) quality and quantity of light.

1. Lighting

Lighting in these phytotrons was supplied by 1) two, 20-watt, two foot long, General Electric,

fluorescent, "cool white" tubes (F20T12.CW) mounted horizontally 12-13 inches above each of four shelves for a total of eight tubes; 2) two, 40-watt, four foot long, Sylvania, Lifeline, fluorescent, "Gro Lux" tubes (F40-Gro) mounted vertically in diagonally opposite corners; and 3)' two, 40-watt, four foot long, Ken Rad fluorescent, "warm white" tubes (F40 WW) mounted vertically in the two remaining diagonally opposite corners.

This lighting mixture produced a wider spectrum of light energies than does any one type of fluorescent tube separately. Two hundred and fifty to 300 ft-c of incident light were produced. Regretfully, a radiospectrometer was not available to record the spectral distribution of light energies produced by this light mixture.

2. Photoperiod

The publications of Kale and Krishnamurthy (1967) and of Kothi (1971) have amply demonstrated that a photoperiod of 16 hrs light-8 hrs darkness produces the optimal vegetative growth in excised pieces of Ulva lactuca var. rigida. All of the investigations reported below were carried out under this photoperiod and the above just described lighting regime.

V. Methods

1. Axenic vs Xenic

Since the cultures producing the inocula are xenic, or more preferably, uni-algal, they are free from any other contaminating algae or fungi. They are, however, not axenic, or "pure", in that they do have associated with them the bacterial flora which was transferred on isolating the initial algal and fungal free fragment.

In a mineral nutrient enrichment supplemented organically only by a very low vitamin level, such as the one used here to produce the inocula, it is not possible for a large bacterial population to develop. This is especially true, when the pieces of U. lactuca are transferred every three to four days into new sterile flasks and medium. Since the work of Provasoli and Pintner (1958) has indicated that axenic cultures of U. lactuca will not develop normally in the enrichments and artificial sea waters now available, it was deemed desirable, for the problem at hand, to have present the bacterial flora associated with U. lactuca and which is evidently responsible and necessary for the normal, natural development of this plant.

This is compatible with the aim of these investigations, namely, the effect of sewage effluents upon

the growth of U. lactuca in the natural habitat with the compliment of bacterial flora as present and actively influencing the growth of this plant. In any case, the number of bacteria introduced on these inocula growing in sterile mineral enrichment is exceedingly small when compared to the number present in the various sewage effluents under investigation.

2. Handling Material Serving as Inocula

In spite of the above statements on the desirability of bacteria being present in the culture in order to secure normal development of the plant, once the isolation had been made which was to serve as the basis through growth of all future inocula, all subsequent manipulations therewith, including the subculturing and the preparing of the inocula, were done under aseptic conditions to the greatest extent possible. This was done to prevent contamination with a different bacterial flora as well as to prevent contamination with fungi and other algae.

3. Culture Vessels

The investigations to follow were carried out in 2800 ml Fernbach flasks containing a final volume of the

test solution of one liter. In this size and type flask, this amount of liquid had a relative large surface area. The mouth of the flask was covered with a loose fitting, 600 ml, low form, Griffin, Pyrex glass beaker. This beaker-cap, while preventing gross contamination, did allow for the free exchange of atmospheric gases, in particular, carbon dioxide (CO_2). Since, in nature, CO_2 is a limiting factor in the growth rates of both terrestrial and aquatic plants, a relatively large surface area and a loose fitting cap are essential to guard against a depressed growth rate caused by limited access to gaseous exchanges, and hence, a CO_2 deficiency. Moreover, these glass beaker-caps, do not interfere with the amount of light, from above, reaching the submerged plants. With large cotton plugs, a significant decrease of the incident light falling on the liquid surface is observed.

4. General Statement on Protocol

Material of U. lactuca to serve as the inocula were grown as described above. Pieces of U. lactuca measuring one by three centimeters (1 cm x 3 cm) or, having thus, an area of three square centimeters (3 cm^2) were cut from this cultivated material. For each of the various solutions tested below, ten flasks were each inoculated with one of these 1 x 3 cm pieces. The

inoculated flasks were shaken once a day to enhance gaseous exchanges. The medium both of the control and of the effluent under investigation was changed every three to four days. At the end of exactly two weeks, the pieces in each flask were measured and an average for the ten flasks was calculated. Also for each experiment an appropriate control consisting of five flasks each was run simultaneously in the same phytotron. At the end of the two week period, these control flasks were also measured and their average growth calculated. From these calculations the effect of the sewage effluents could be numerically defined and considered.

VI. Chemical Analyses of Sewage Effluents under Investigation

In the experiments to follow, the actual vegetation growth realized in the various sewage effluents and their respective controls could be physically measured, numerically defined, and considered. However, this would not tell us what particular entity or entities in the sewage effluents were responsible for any increased growth over the control which may be observed.

For each of the sewage effluents investigated, a 500 ml sample of the liquid being tested was prepared

and sent to the Ecology-Pollution Analysis Laboratory for analyzation of the major chemical compounds present. It was hoped that through these analyses, the exact causative agent or agents in sewage which promote the measurable growth of U. lactuca could be ellucidated. The results of these analyses are given in Table I. These will be further discussed elsewhere in this report.

VII. Experiments

1. Establishing the Optimal Parameters of Growth

A. Salinity over the Wide Range

Because many areas which support luxuriant populations of U. lactuca are located in brackish waters, it was necessary to determine the effect of salinity on the optimal growth of this alga when all other growth parameters were constant.

To realize this, ten flasks for each of the following percentages of sea water were prepared. In order to assure that the nutrient levels remain the same at each salinity, both the sea water and the distilled water, which was used to dilute the seawater to the needed percentages, were enriched identically with N_2M (see Appendix II for its preparation). Thus, regardless of

the proportions in which these were mixed, the nutrient level would remain constant while only the salinity would vary. The salinities tested are:

per cent sea water	salinity ‰
100	30.0
75	22.5
50	15.0
25	7.5
0	0.0

Each of these 50 flasks were inoculated with a single 3 x 1 cm strip of U. lactuca. These flasks were then incubated for two weeks at 25°C under a 16 hrs light-8 hrs darkness photoperiod. The flasks were shaken daily to allow better gaseous exchanges. Every three to four days the strips of U. lactuca were transferred to sterile flasks containing sterile media at identical salinities and at identical nutrient levels. This was done to prevent any hinderance of growth due to 1) the exhaustion of nutrient supply or 2) the production and build up of metabolic toxins or inhibitors in the aging media. At the end of exactly two weeks, these 50 strips were measured. The results are recorded in Table II.

B. Salinity over the Narrow Range

From Table II it can be readily observed that best growth occurred at 21‰ or in 75% of the salinity of high salinity sea water. However, this determination does not rule out the possibility that even a better growth rate might occur at a somewhat higher or lower salinity. To explore these possibilities, the experiment was repeated just as previously described, but at the following salinities:

per cent sea water	salinity
80	24.4
75	21.0
70	19.6

The results of this experiment are recorded in Table III.

C. Temperature

Working with a photoperiod of 16 hrs light-8 hrs darkness and with salinity of 21 or 75% that of high salinity sea water as given the optimal growth of U. lactuca, it was necessary to establish what temperatures produced optimal growth. For each of the seven temperatures listed below, ten flasks were inoculated

with a 3 x 1 cm strip of U. lactuca, each flask containing one liter of 75% seawater enriched with N_2M.

Degrees Centigrade:	
35	15
30	10
25	5
20	

These 70 flasks were incubated and handled essentially as those of the preceding experiments. The results of these experiments are recorded in Table IV.

D. Effect of Various, Common Enrichments of Sea water on the Growth of U. lactuca

For the artificial cultivation of marine algae under laboratory controlled conditions, there have been developed a number of enrichments which promote the growth of various marine algae. In order to determine the magnitude of growth that some of these may have on U. lactuca, the following enrichments were tested:

1. Erdschreiber (see Appendix I for preparation)
2. N_2M (see Appendix II for preparation)
3. Artificial Sea water (see Appendix III for preparation)

4. Ott's Enrichment (see Appendix IV for preparation)
5. Un-enriched Sea water (Control)

For each of these solutions, ten flasks each containing one liter of solution were inoculated with a 3 x 1 cm strip of U. lactuca. These 50 flasks were incubated for two weeks at 25°C under a 16 hrs light-8 hrs darkness photoperiod. The flasks were shaken daily to allow better gaseous exchanges. Every three to four days the strips of U. lactuca were transferred to new sterile flasks containing sterile, identical media. At the end of exactly two weeks these 50 strips were measured. The results are recorded in Table V.

E. Brief Summary of the Experiments to Determine the Optimal Parameters of Growth in U. lactuca

From the above three basic preliminary experiments it has been determined:

1) That best growth occurs in 21‰ or ca. 75% that of high salinity sea waters.
2) That best growth occurs at a temperature of 25°C.
3) That all enrichments of natural sea waters containing nitrate and phosphates do not enhance the growth of U. lactuca to the same extent,

and that, therefore, other growth promoting factors may be operative.

From the literature, it has been shown that a photoperiod of 16 hrs light-8 hrs darkness produces the best vegetative growth in U. lactuca. With these basic parameters of growth established and shown applicable to the material present in the Elizabeth River, it is now possible to test, with a degree of assurance, the effect of sewage effluents and their derivatives upon the growth of U. lactuca.

2. Effect of Sewage Effluents upon the Growth

A) Effect of Sewage Effluents at 1% Levels

1. Sewage effluents for these experiments were collected at the James River Plant in the Hampton-Newport News area.

2. The high salinity sea water to which these sewage effluents were added was collected at Wachapreague, Virginia, and has a salinity of ca. 30 before dilution.

The first set of experiments utilizing sewage effluents follows exactly the recommended concentrations of the consultant, Dr. Clair N. Sawyer.

The following listed sewage effluents were added to high salinity sea water, which had been diluted to

the 75% salinity by the addition of distilled water, to give a final sewage effluent concentration of 1%:

1. raw sewage
2. settled sewage
3. un-nitrified sewage
4. nitrified sewage

For each of these four types, ten flasks each containing one liter of the individual effluent were inoculated with a 1 x 3 cm strip of U. lactuca. The flasks were incubated under the derived, optimal, environmental conditions just discussed. These flasks were shaken once a day. Every three to four days the strips of U. lactuca were transferred to new flasks each containing one liter at the same concentrations (1%) of the respective sewage effluents. At the same time, ten flasks of high salinity, un-enriched sea water were inoculated and simultaneously incubated in the same phytotron. These ten flasks served as a control for this experiment. At the end of exactly two weeks these 50 strips of U. lactuca were measured. The results are recorded in Table VI.

B) Effect of Sewage Effluents at 10% levels

From Table V, it is evident that best growth occurred in raw and settled sewage. However, the growth was not as great as had been realized in enriched sea waters without the addition of sewage effluents, nor was the growth sufficient, in my opinion, to produce luxuriant populations of U. lactuca. Because of this, it was decided that this experiment should be repeated, but at 10% sewage effluent levels. Again a control set of ten flasks were simultaneously incubated in the same phytotron. Ten flasks of each of the four sewage types at 10% concentration were inoculated and incubated just as previously described for the 1% concentration. At the end of exactly two weeks, these 50 strips of U. lactuca were measured. The results of this experiment are recorded in Table VII.

VIII. Discussion and Summary

1. The optimal growth parameters of U. lactuca have been established:

   A. Temperature. U. lactuca grows best at 25°C when the other parameters are held constant. This relative high temperature for a marine organism is compatible with the observations

made in the field that high, surface, water temperatures accompany and promote luxuriant growth of this plant.

B. Photoperiod. A photoperiod of 16 hrs light-8 hrs darkness produces the best vegetative growth of U. lactuca. Again this is compatible with the luxuriant growth of this plant during the summer months when the duration of light is the longest.

C. Salinity. A salinity of ca. 21 gives the best vegetative growth rate and is compatible with field observations that luxuriant growth of U. lactuca often occurs in brackish water areas.

D. These three important growth parameters of 1) temperature, 2) photoperiod, and 3) salinity when operative simultaneously will double the size of U. lactuca every three to four days in N_2M medium. This is a growth rate comparable to that reported by Kanwisher (1966) for U. lactuca at Woods Hole, Massachusetts, a non-polluted area.

2. The effect on growth of raw, settled, un-nitrified and nitrified sewage has been established:

A. Using genetically and physiologically uniform

material artificially grown under the above described, controlled conditions, it has been demonstrated, for both 1% and 10% concentrations of sewage effluents, that settled and raw sewage have a stimulating effect on the growth of U. lactuca, with the greater growth rates achieved in 10% concentrations.

B. Nitrified and un-nitrified sewage effluents, at 1% and 10% concentration, do not stimulate growth in U. lactuca over that of the control. It appears that these type of effluents may even cause a slight inhibitory effect.

CHEMICAL ANALYSES

	DKN (mg/l)	NH_3 (mg/l)	DP ($\mu gAt^P/l$)	NO_2 ($\mu gAt^N/l$)	NO_3 ($\mu gAt^N/l$)	$O\text{-}PO_4$ ($mgAt^P/l$)
75% SW	1.680	1.019	2.670	2.45	3.54	1.62
75% SW + 1% RS	3.750	3.335	7.350	4.63	5.88	1.16
75% SW + 1% SS	1.512	1.333	7.800	4.73	5.93	3.94
75% SW	0.596	0.062	0.946	0.55	2.39	0.16
75% SW + 1% NS	0.2044	0.176	3.527	0.90	3.31	2.89
75% SW + 1% UNS	0.4648	0.160	3.673	0.52	3.54	2.83
75% SW	0.8990	0.812	2.000	1.37	5.07	1.19
75% SW + 10% RS	3.360	2.201	38.000	4.91	0.82	32.32
75% SW + 10% SS	4.984	2.800	36.910	4.91	3.10	36.64
75% SW	4.2560	2.470	1.539	1.04	3.09	0.76
75% SW + 10% NS	3.1920	2.860	31.398	0.64	3.39	25.80
75% SW + 10% UNS	4.1440	4.032	31.999	1.28	4.96	35.64

SW = Sea water
RS = Raw sewage
SS = Settled sewage
NS = Nitrified sewage
UNS = Unnitrified sewage
DKN = Dissolved Kjeldahl nitrogen
NO_3 = Nitrate
NH_3 = Ammonia
DP = Dissolved phosphorus
NO_2 = Nitrite
$O\text{-}PO_4$ = Ortho-phosphate

Table II: Effect of Salinity Over the Wide Range

% of High Salinity Sea Water	Salinity (o/oo)	Growth of 3 X 1 cm Inocula After Exactly Two Weeks					Average Size in 2 Weeks	Total Size in cm^2	Actual Increase in cm^2	Percent Increase
		Flask								
		1	2	3	4	5				
100	30.0	5.0 X 2.0	5.0 X 1.7	5.7 X 2.0	6.0 X 2.0	5.5 X 2.5	5.4 X 2.0	10.80	7.80	260.0
75	22.5	7.2 X 2.7	7.8 X 3.0	6.4 X 2.2	8.0 X 2.7	7.9 X 3.1	7.5 X 2.7	20.25	17.25	575.0
50	15.0	5.0 X 2.7	4.6 X 1.8	5.5 X 1.8	5.1 X 1.7	5.1 X 1.7	5.0 X 1.7	8.50	5.5	183.3
25	7.5	4.3 X 1.3	4.4 X 1.4	4.0 X 1.2	4.2 X 1.6	4.6 X 1.5	4.5 X 1.4	6.02	3.02	100.0
0	0.00	all perished					—	—	—	—

Table II

Table III: Effect of Salinity Over the Wide Range

% of High Salinity Sea Water	Salinity (o/oo)	Growth of 3 X 1 cm Inocula After Exactly Two Weeks					Average Size in 2 Weeks	Total Size in cm^2	Actual Increase in cm^2	Percent Increase
		Flask								
		1	2	3	4	5				
80	24.0	4.1 X 1.2	4.2 X 1.2	3.2 X 1.1	3.0 X 1.0	4.0 X 1.4	3.7 X 1.1	4.07	1.07	35.6
75	22.5	4.3 X 1.5	4.2 X 1.3	4.3 X 1.8	4.2 X 1.7	3.5 X 1.1	4.5 X 1.4	6.3	3.3	110.0
70	21.0	4.4 X 1.3	3.0 X 1.1	3.7 X 1.5	3.0 X 1.0	3.0 X 1.0	3.4 X 1.1	3.74	0.74	24.6

Table IV: Effect of Temperature

Temperature in Centigrade	Growth of 3 X 1 cm Inocula After Exactly Two Weeks					Average Size in 2 Weeks	Total Size in cm^2	Actual Increase in cm^2	Percent Increase
	Flask								
	1	2	3	4	5				
35	3.5 X 1.2	3.1 X 1.0	3.2 X 1.1	3.0 X 1.0	3.2 X 1.1	3.2 X 1.0	3.20	0.20	6.6
30	4.3 X 1.5	4.2 X 1.3	4.3 X 1.8	4.2 X 1.7	3.8 X 1.4	4.3 X 1.6	6.88	3.88	129.3
25	7.2 X 2.7	7.8 X 3.0	6.4 X 2.2	8.0 X 2.7	7.9 X 3.1	7.5 X 2.7	20.25	17.25	575.0
20	5.5 X 1.5	4.0 X 1.1	3.6 X 1.2	9.7 X 3.0	7.2 X 2.5	6.0 X 1.8	10.80	7.80	260.0
15	3.5 X 1.1	5.0 X 1.2	4.2 X 1.1	3.6 X 1.2	6.0 X 1.6	4.5 X 1.2	5.40	2.40	80.0
10	5.2 X 1.5	3.7 X 1.2	3.7 X 1.1	3.5 X 1.2	6.0 X 1.8	4.4 X 1.3	5.72	2.72	90.2
5	3.5 X 1.2	4.0 X 1.5	4.2 X 1.5	3.5 X 1.5	4.0 X 1.3	3.8 X 1.1	4.18	1.18	39.3

Table V: Effect of Various Sea Water Enrichments

Enrichment	Growth of 3 X 1 cm Inocula After Exactly Two Weeks (Flask 1)	(Flask 2)	(Flask 3)	(Flask 4)	(Flask 5)	Average Size in 2 Weeks	Total Size in cm^2	Actual Increase in cm^2	Percent Increase
N_2M Enrichment	5.0 X 1.6	4.2 X 1.3	4.8 X 1.7	5.7 X 1.5	6.0 X 2.1	5.1 X 1.6	8.16	5.16	172.0
Unenriched Sea Water	5.2 X 1.8	5.5 X 1.8	5.5 X 2.0	4.8 X 1.8	5.3 X 1.8	5.3 X 1.8	9.54	6.54	218.0
Ott's Enrichment	9.9 X 3.4	8.8 X 3.0	7.5 X 2.5	8.3 X 3.0	8.3 X 3.0	8.6 X 3.0	25.80	22.80	760.0
Artificial Sea Water	4.6 X 1.6	4.0 X 1.3	4.9 X 1.5	4.6 X 1.2	4.6 X 1.5	4.5 X 1.4	6.30	3.30	110.0
Erdschreiber Enrichment	6.8 X 2.5	7.0 X 2.6	6.6 X 2.3	7.0 X 2.6	7.1 X 2.6	6.9 X 2.5	17.25	14.25	475.0

Table VI: Effect of 1% Sewage Effluents

Sewage Effluent	Growth of 3 X 1 cm Inocula After Exactly Two Weeks — Flask 1	2	3	4	5	6	7	8	9	10	Average Size in 2 Weeks	Total Size in cm^2	Actual Increase in cm^2	Percent Increase
RS	7.3 X 2.0	5.8 X 2.2	5.6 X 2.0	6.0 X 1.6	5.8 X 1.8	5.0 X 2.1	5.0 X 2.0	6.0 X 2.1	6.0 X 1.6	6.5 X 2.5	5.9 X 1.9	11.21	8.21	273.6
SS	5.8 X 2.1	6.1 X 2.0	5.0 X 2.0	5.1 X 2.1	4.3 X 2.3	5.3 X 2.1	6.0 X 2.0	5.3 X 1.9	5.3 X 2.1	6.0 X 2.3	5.6 X 2.0	11.20	8.20	273.3
UES	5.7 X 2.0	5.0 X 1.6	5.3 X 1.6	5.1 X 1.8	5.2 X 1.5						5.3 X 1.7	9.01	6.01	200.0
NS	3.5 X 1.1	4.0 X 1.5	3.9 X 1.2	3.5 X 1.3	3.6 X 1.2	3.5 X 1.3	3.6 X 1.2	3.5 X 1.2	3.2 X 1.1	3.0 X 1.1	3.5 X 1.2	4.20	1.20	40.0
UNS	3.2 X 1.3	3.7 X 1.3	3.0 X 1.1	4.8 X 1.4	3.0 X 1.0	3.1 X 1.1	3.1 X 1.1	3.6 X 1.2	3.5 X 1.1	3.6 X 1.5	3.4 X 1.2	4.08	1.08	36.0
UES	3.5 X 1.2	3.0 X 1.2	3.8 X 1.1	3.3 X 1.3	3.3 X 1.2						3.5 X 1.2	4.20	1.20	40.0

RS = Raw sewage
SS = Settled sewage
UES = Unenriched seawater
NS = Nitrified sewage
UNS = Unnitrified sewage

Table VII: Effect of 10% Sewage Effluents

Sewage Effluent	Growth of 3 X 1 cm Inocula After Exactly Two Weeks — Flask 1	2	3	4	5	6	7	8	9	10	Average Size in 2 Weeks	Total Size in cm^2	Actual Increase in cm^2	Percent Increase
RS	8.0 X 4.5	7.2 X 2.1	10.0 X 3.5	6.1 X 2.0	6.7 X 2.1	6.4 X 1.5	6.4 X 2.6	7.8 X 3.5	9.4 X 2.4	8.0 X 4.2	7.6 X 2.8	21.28	18.28	609.3
SS	8.1 X 3.1	7.9 X 2.8	7.0 X 2.2	7.8 X 2.5	5.0 X 1.6	7.6 X 3.5	8.4 X 2.4	7.0 X 2.8	7.5 X 1.9	5.3 X 1.6	7.1 X 2.4	16.04	13.04	434.6
UES	4.5 X 1.3	5.0 X 2.1	4.3 X 1.3	5.0 X 1.7	5.0 X 1.7						4.6 X 1.6	7.36	4.36	145.3
NS	4.6 X 1.7	4.3 X 1.5	4.1 X 1.2	4.4 X 1.5	3.3 X 1.2	3.6 X 1.5	3.5 X 1.0	3.2 X 1.3	4.6 X 1.5	4.0 X 1.2	3.9 X 1.3	5.07	2.07	69.0
UNS	3.7 X 1.3	3.8 X 1.5	3.7 X 1.2	4.5 X 1.3	4.0 X 1.2	3.2 X 1.2	3.4 X 1.2	3.2 X 1.2	3.3 X 1.2	3.6 X 1.3	3.6 X 1.2	4.32 6.02	1.32	44.0
UES	4.3 X 1.2	5.2 X 1.8	4.1 X 1.4	4.2 X 1.4	4.0 X 1.5						4.3 X 1.5	6.02	3.02	100.0

RS = Raw sewage
SS = Settled sewage
UES = Unenriched seawater
NS = Nitrified sewage
UNS = Unnitrified sewage

X.

APPENDIX I

The Preparation and Composition of Erdschreiber's Solution

The following is Föyn's (1934) modification of Schreiber's (1928) solution, and, it has long been a standard enrichment of natural sea water when used for the cultivation of marine algae:

Sea water	1000.00 ml
$NaNO_3$	0.20 gr.
Na_2HPO_4	0.02 gr.
Soil extract	50.00 ml

The soil extract (Bold, 1942) is prepared by adding one part garden soil to two parts of distilled water and steaming in an Arnold Sterilizer for one hour on each of two consecutive days. This mixture is allowed to settle, is then decanted, and only the clear supernatant is used. If necessary, the supernatant is filtered through a No. 1 Whattman filter paper.

The success of this enrichment is due primarily to the soil extract. Care must be taken in choosing the right type of soil. Good, rich garden soil which has not recently been fertilized is preferable. Several batches of soil may have to be tried before a satisfactory

growth rate of the algae can be realized.

APPENDIX II

The Preparation and Composition of the Enrichment N_2M

N_2M is an enrichment of natural sea waters which permits the mass cultivation of a number of phytoplanktonic organisms serving as a food source for a variety of zoological taxa. This enrichment is added to filtered sea water *via* a single stock solution, the composition of which is as follows:

1) Sodium silicate solution	100 ml
2) Ketchum and Redfield's Solution "A"	200 ml
3) Ketchum and Redfield's Solution "B"	100 ml
4) Sodium Molybdate Solution	50 ml
5) Arnon's Micronutrient Solution (mod.)	50 ml
6) Soil Extract	200 ml

The above mixture is dispensed into bottles, plugged, sterilized, and stored in a refrigerator at *ca*. 0°C. It is routinely used at the rate of 2 ml per liter of sea water.

The resulting, enriched seawater is sterilized before use. To prevent the pH of the mass cultures from reaching dangerously high levels HCl is added, usually at the rate of 2 ml of 0.1N HCl per liter of

final medium. It is added before sterilization.

For certain species of algae, it has been found advantageous to add a vitamin mix which gives the following concentrations in the final medium (Dr. R. R. L. Guillard, Personal Communication):

Thiamin HCl	0.2 mg
Biotin	1.0 µg
B_{12}	1.0 µg

The preparation of the single stock solution for the N_2M enrichment as given above requires 6 stock solutions; their preparation is as follows:

1) Sodium Silicate Solution

$Na_2SiO_3.9H_2O$	4.66 g
Dist. H_2O	to 100 ml

2) Ketchum and Redfield's Solution "A"

KNO_3	20.2 g
Dist. H_2O	to 100 ml

3) Ketchum and Redfield's Solution "B"

$Na_2HPO_4.7H_2O$	3.0 g
$CaCl_2.2H_2O$	2.7 g
$MgSO_4$ (anhyd.)	2.9 g
$FePO_4$	0.5 g
HCl (conc.)	2.0 ml
Dist. H_2O	to 100 ml

4) Sodium Molybdate Solution

$Na_2MoO_4 . 2H_2O$	0.0119 g
Dist. H_2O	to 100 ml

5) Arnon's Micronutrient Solution (mod.)

H_3BO_3	0.286 g
$MnCl_2 . 4H_2O$	0.181 g
$ZnSO_4 . 7H_2O$	0.0222 g
$CuSO_4 . 5H_2O$	0.0079 g
$CoCl_2 . 6H_2O$	0.004 g
Dist. H_2O	to 100 ml

6) Soil Extract Solution

This solution must be made up immediately before mixing into the N_2M stock solution.

Mix 1 kilogram of top soil with 2 liters of distilled water and sterlize in a large flask 1 hour at 15 lbs. pressure. Allow to settle for several days, decant the liquid, pass it through a layer of cotton, then filter through a Whatman No. 1 filter paper. The soil extract is now ready to be used (Bold, 1942).

APPENDIX III

Preparation and Composition of Artificial Sea Water (U-1)

For each liter of artificial sea water needed, dissolve the below indicated amounts of NaCl and $MgSO_4 \cdot 7H_2O$ individually in separate 200 ml volumes of distilled water. When dissolved, add these to 500 ml of distilled water mixing well after addition. Then add the ten other liquid volumes, individually, and mix well after each addition. When all components have been added, dilute to exactly one liter, mixing well. Sterilize before use.

1) NaCl	25.00 gr
2) $MgSO_4 \cdot 7H_2O$	8.00
3) 10% solution of KCl	7.00 ml
4) 10% solution of $CaCl_2 \cdot 2H_2O$	2.80
5) 1% solution of $NaSiO_3 \cdot 9H_2O$	7.00
6) 1% solution of $NaNO_3$	20.00
7) 1% solution of KH_2PO_4	0.50
8) Micronutrients[A)]	1.00
9) 1[S] metal mix[B)]	0.50
10) "Tris" Buffer[C)]	4.00
11) Guillard's 3-vitamin mix[D)]	1.00
12) Eagle vitamin mix[E)]	10.00

A) Micronutrients (modified from Provasoli, McLaughlin, and Droop, 1957). Dissolve the below listed compounds in the amounts indicated, individually, each in ca. 45 ml of distilled water. Pour singly into a 500 ml volumetric flask, shaking well after each addition. When all have been added and mixed, dilute with distilled water to exactly 500 ml.

1) Na_3 versenol.$2H_2O$	16.50
2) $FeCl_3.6H_2O$	4.84
3) $ZnCl_2$	0.525
4) $MnCl_2.4H_2O$	1.80
5) $CoCl_2.6H_2O$	0.02
6) $CuCl_2.2H_2O$	0.027
7) H_3BO_3	5.72
8) $Na_2MoO_4.2H_2O$	0.63
9) V_2O_3	0.018
10) As_2O_3	0.0495

B) 1^S metal mix (Provasoli, McLaughlin, and Droop, (1957). Dissolve the below listed compounds in the amounts indicated, each individually in ca. 50 ml of distilled water. Pour singly into a 500 ml volumetric flask containing ca. 150 ml of distilled water. Mix well after each addition.

When all have been added, dilute to exactly 500 ml.

1) $SrCl_2.6H_2O$	25.805 gr
2) $AlCl_3.6H_2O$	2.24
3) RbCl	0.14
4) LiCl	0.615
5) KI	0.035
6) NaBr	41.925

C) To 100 ml of distilled water, add 50 grs of "Tris" (Tris (Hydroxymethyl) amino-methane) and dissolve. Then add carefully, 5 ml at a time, conc. HCl until a pH of 7.2 is reached. As pH 7.2 is reached, add the remaining needed acid drop-wise until pH 7.2 is reached. Then dilute to exactly 200 ml with distilled water. 2 ml = 500 mg of "Tris."

D) Guillard's 3-vitamin mix (Guillard, 1961). To 100 ml of distilled water add and dissolve:

Biotin	0.1 mg
B_{12}	0.1 mg
Thiamin.HCl	20.1 mg

This stock solution is dispensed in 4 ml lots into screw-capped test tubes, and sterilized. Keep frozen until used.

E) Eagle Vitamins (purchased from Difco Laboratories, Detroit, Michigan). Eagle Vitamins, "Bacto-TC Vitamins, Minimal, Eagle, dried" are purchased in dried form, 100 mg per vial. This dried powder contains the following vitamins in the concentration indicated:

1) Choline chloride	1.0 mg
2) Folic Acid	1.0
3) Inositol	2.0
4) Nicotinamide	1.0
5) Calcium panthothenate	1.0
6) Pyridoxal	1.0
7) Riboflavin	0.1
8) Thiamine Hydrochloride	1.0

The contents of one vial, or 100 mg, are dissolved in 100 ml of distilled water by heating to the boiling point. When solution has been achieved, sterilize by millipore filtration. This is used in our artificial sea waters at the rate of 10 ml per liter of sea water. When used at this rate, the vitamin concentration per liter of sea water is one tenth (0.1) that given above.

APPENDIX IV

Preparation and Composition of Ott's Enrichment of Natural Sea Water

The following medium is my modification of Provasoli's (1962) "ES Enrichment" and of Guillard's (1961) "f medium." It has the distinct advantage over the classical "Erdschreiber" (Föyn, 1934) in that the soil-extract is replaced by a vitamin mix and chelated micronutrients of known concentration, thus permitting a high degree of reproducibility in most laboratories.

This medium when prepared according to the schedule given below produces a clear, precipitate-free medium:

I. To 4000 ml of filtered sea water, add the following, and boil (or sterilize):
   (1) 80 ml of 1% $NaNO_3$
   (2) 1.6 ml of tris (Hydroxymethyl) aminomethane, (Tris Buffer), stock solution at the concentration of 250 mg/ml and adjusted to pH 7.3 with HCl, (should be used only with aeration of cultures); this is also toxic to some algae.

II. To 50 ml of distilled water, add the following, and boil (or sterilize):

(1) 4 ml each of micronutrient solutions Nos. 1, 2, 3, and 4. (See A below for their preparation).

(2) 4 ml of FeEDTA, 1 ml = 1 mg of Fe, (EDTA = Ethylene - diaminetetraacetic acid), (see B below for its preparation).

III. To 50 ml of distilled water add the following, and boil (or sterilize):

(1) 10 ml of 1% sodium glycerophosphate

IV. When (I) above has cooled, add II and III above, and 4 ml of the 3-vitamin stock solution, (see C below for its preparation), mix well.

A. Micronutrients: These have been modified from those presented by Deason and Bold (1960) in that the $CuSO_4$ is omitted in micronutrient solution No. 2, and the designations of the stocks have been changed.

		g/l
(1)	$FeSO_4.7H_2O$	4.98
	$ZnSO_4.7H_2O$	8.82
	$MnCl_2.4H_2O$	1.44
(2)	MoO_3	0.71
	$Co(NO_3)_2.6H_2O$	0.49

(3)	EDTA	50.0
	KOH	31.0
(4)	H_3BO_3	11.42

B. FeEDTA: This is prepared by the method of Provasoli (1962) but at 10 times the concentration thereof.

To 500 ml of distilled water add, and dissolve:

$Fe(NH_4)_2(SO_4)_2.6H_2O$	3.51 g
NaEDTA	3.30 g

C. 3-Vitamin Stock Solution: The following is modified from Guillard (1961).

To 100 ml of distilled water add:

Biotin	0.1 mg
B_{12}	0.1 mg
Thiamin. HCl	20.0 mg

This stock solution is dispensed in 4 ml lots in screwcapped test tubes, and sterilized. It then should be kept frozen until used.

XI. RECOMMENDATIONS

1) The preliminary results presented in this report dealing with sewage effluents should be verified utilizing sewage effluent from another sewage treatment plant.

2) Effluent from a pilot plant utilizing the treatment techniques expected to be employed at HRSDC primary plants in the Elizabeth should be tested.

3) If stimulation results from the pilot plant, effluent bioassays to identify the growth stimulating compound or compounds will be necessary.

XII. LITERATURE CITED

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